In vitro culture of human embryos : effects on fetal development and the role of the placenta

In vitro culture of human embryos

Effects on fetal development and the role of the placenta

Ewka Nelissen

ISBN: 978-90-6464-854-0

Financial support for the publication of this thesis has been kindly provided by:

Goodlife Pharma BV, Stichting Fertility Foundation, Organon/MSD, Medical Dynamics, Bayer B.V., BMA B.V. (Mosos), Grafimedics, Chipsoft B.V.

Cover: Image from Lennart Nilsson / TT
Layout: Ferdinand van Nispen, Citroenvlinder-dtp.nl, Bilthoven, The Netherlands
Print: GVO drukkers & vormgevers B.V. | Ponsen & Looijen, Ede, The Netherlands

In vitro culture of human embryos

Effects on fetal development and the role of the placenta

PROEFSCHRIFT

ter verkrijging van de graad van doctor aan de Universiteit Maastricht,
op gezag van de Rector Magnificus, Prof. dr. L. Soete,
volgens het besluit van het College van Decanen,
in het openbaar te verdedigen
op donderdag 26 februari 2015 om 16.00 uur

door

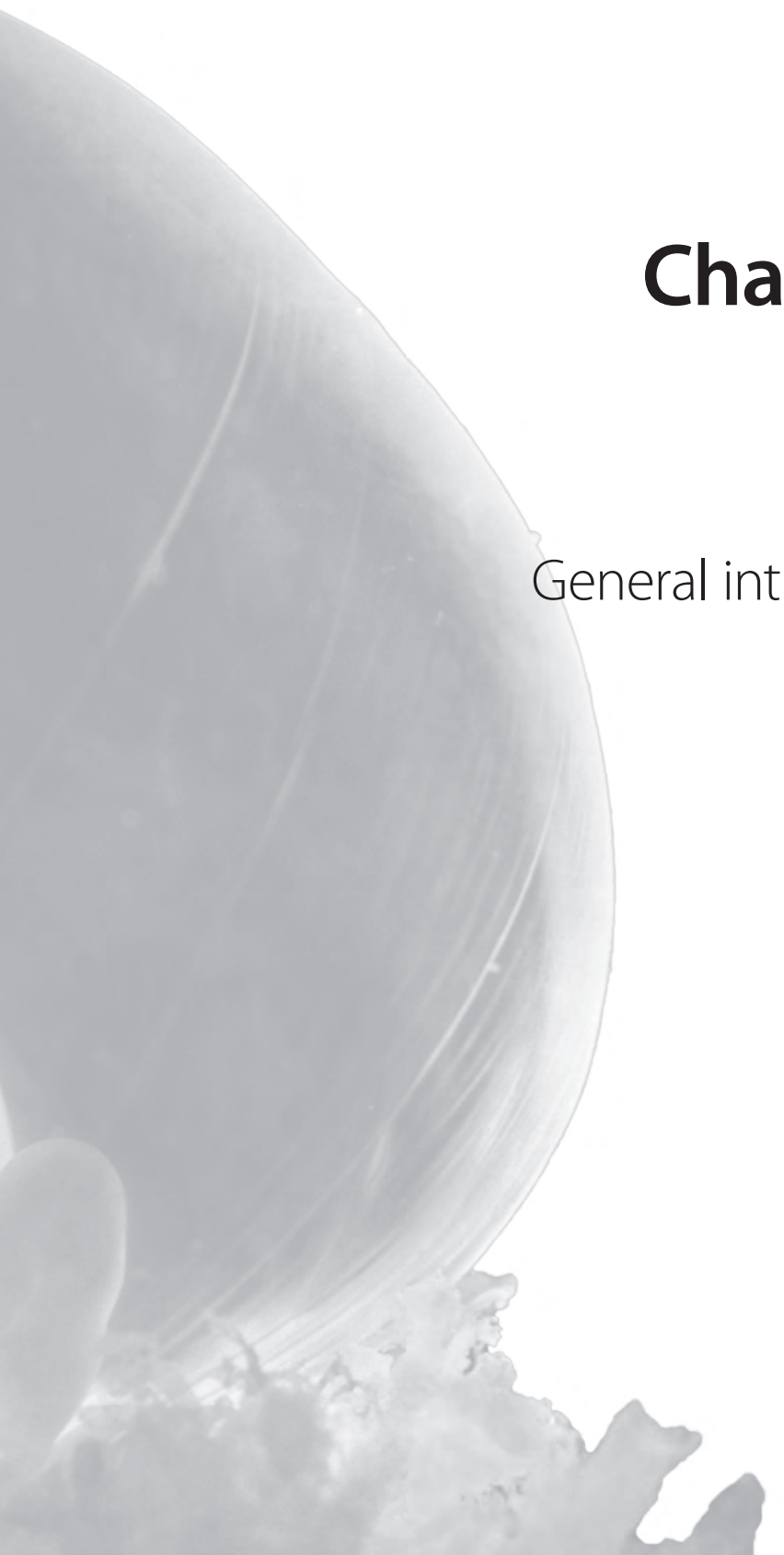
Ewka Catharina Matthea Nelissen

Promotor
Prof. dr. J.L.H. Evers

Copromotoren
Dr. J.C.M. Dumoulin
Dr. A.P.A. van Montfoort

Beoordelingscommissie
Prof. dr. C.E.M. de Die-Smulders (voorzitter)
Prof. dr. J.J.H.M. Erwich (UMC Groningen)
Prof. dr. S. Repping (AMC Amsterdam)
Prof. dr. M.E.A. Spaanderman
Prof. dr. L.J.I. Zimmermann

Contents



Chapter 1

General introduction

General introduction

Worldwide, the use of assisted reproduction technology (ART) has risen substantially and it is estimated that around 5.5 million babies have been born since the first IVF baby in 1978. In the latest world report, from 2006, an estimated number of 256 668 infants were born that year (Mansour *et al.*, 2014). The most recent report from the European Society of Human Reproduction and Embryology covering 2009 showed that the percentage of infants conceived through ART varied from 0.6% of national births in Moldova to 4.5% in Denmark, with several countries exceeding 3% (Ferraretti *et al.*, 2013). These figures underline that the proportion of children conceived through ART is not negligible and will influence the coming generations. Hence, it is important to monitor the consequences of manipulating gametes and embryos during ART.

The adverse perinatal outcome after ART is well-known. Elective single embryo transfer (eSET) has to a large extent abolished the problems associated with multiple pregnancies. However, eSET is not the only solution to the adverse perinatal outcome, as growing evidence has shown that even in singletons, ART is associated with an increased risk of preterm birth, low birthweight, very low birthweight, hypertensive disorders of pregnancy, antepartum haemorrhage, premature rupture of membranes, caesarean section, congenital malformations and cerebral palsy, when compared with singletons from spontaneous conceptions (Helmerhorst *et al.*, 2004; Jackson *et al.*, 2004; McGovern *et al.*, 2004; McDonald *et al.*, 2009; Kallen *et al.*, 2010a; Kallen *et al.*, 2010b; Kallen *et al.*, 2010c; Sazonova *et al.*, 2011; Pandey *et al.*, 2012).

After all these years of ART use, the origin of this adverse perinatal outcome is still not fully understood. It may be due to intrinsic patient factors or to the technical procedures, including hormonal stimulation, culture media composition, duration of embryo culture, IVF/ICSI technique and freezing/thawing techniques. A major concern is whether the IVF techniques themselves could have negative impacts on the ART offspring. A recent systematic review by Pinborg *et al.* (2013), which aimed to elucidate why singletons conceived after ART have this poorer outcome, demonstrated that both subfertility and ART play an important role, although it is still not totally clear which (part of the) technique is responsible.

From animal studies it is clear that culture medium constituents are responsible for birthweight changes in offspring. Various culture conditions,

mainly associated with the addition of serum, have led to the so-called large offspring syndrome in sheep and cattle (Thompson *et al.*, 1995; Young *et al.*, 1998; Numabe *et al.*, 2000; Sinclair *et al.*, 2000; Rooke *et al.*, 2007; Camargo *et al.*, 2010) and to a reduced birthweight in mice (Khosla *et al.*, 2001). Also other types of *in vitro* stress upon the preimplantation embryo like a decrease in pH (Zander-Fox *et al.*, 2010), accumulating ammonium (Lane *et al.*, 1994; Zander *et al.*, 2006) or different oxygen conditions (Feil *et al.*, 2006) can result in aberrant fetal growth in ART offspring. In human ART there is still little knowledge on the effect of culture medium type or any other type of *in vitro* stress, on birthweight, while the variation in types of culture media is large. Moreover, it is unknown which commercially available embryo culture medium leads to the best IVF/ICSI success rate, as was concluded in a recent systematic review after evaluation of 31 different culture medium comparisons from twenty-two RCTs (Mantikou *et al.*, 2013).

We wondered whether culture medium could also be one of the causes of the adverse perinatal outcome reported after ART in humans. This has led to our first research question, namely if *in vitro* culture medium for human embryos affects perinatal outcome. In the studies described in the first part of this thesis, we investigate the effects of culture medium on human fetal growth and birthweight.

Furthermore, we are interested in the possible etiology. Epigenetic marks, deposited early in development, can respond to intrinsic and environmental stimuli and adapt accordingly throughout life. They regulate the phenotype through the regulation of gene expression by mechanisms other than changes in the underlying DNA sequence. At the same time ART procedures are applied, a crucial epigenetic reprogramming takes place in the embryo. The parental genomes undergo two waves of genome-wide demethylation and remethylation at a vulnerable time window where stochastic and/or environmentally (i.e. ART) induced epigenetic deregulation may occur. The first round of epigenetic reprogramming occurs in the germ line, while the second occurs after fertilization (see Figure 1). Imprinted genes, which are expressed in an epigenetically induced parent-of-origin-dependent way, are protected from reprogramming during the second round, to preserve the parental imprints in the developing embryo (Reik *et al.*, 2001; van Montfoort *et al.*, 2012). Currently, fertilization and embryo culture take place in media which are not optimized for human embryo development, creating a suboptimal environment during this

critical phase. Moreover, it is known that epigenetic modifications are sensitive to the environment (Fraga *et al.*, 2005; Jirtle *et al.*, 2007). Therefore, the poorer outcome after ART could be related to epigenetic disturbances.

Animal models have shown that preimplantation embryo culture can induce epigenetic changes in embryos of sheep (Young *et al.*, 2001), cattle (Lonergan *et al.*, 2006) and mice (Sasaki *et al.*, 1995; Doherty *et al.*, 2000; Khosla *et al.*, 2001; Mann *et al.*, 2004; Li *et al.*, 2005; Fauque *et al.*, 2007; Fernandez-Gonzalez *et al.*, 2009; Market-Velker *et al.*, 2010; Schwarzer *et al.*, 2012). In the placenta, imprinted genes are abundantly expressed and essential for normal placental development and physiology (Frost *et al.*, 2010). Furthermore, animal models have shown that the placenta is much more vulnerable to preimplantation epigenetic disturbance than embryonic tissues (Mann *et al.*, 2004; Rivera *et al.*, 2008; Fauque *et al.*, 2010). This may lead to abnormal placental development and function. Deregulation of placentation can lead to adverse outcomes for both mother and fetus, e.g. preeclampsia and fetal growth retardation (James *et al.*, 2010; Mouillet *et al.*, 2010; Steegers *et al.*, 2010). Altogether, this has led to our second research question, namely whether ART has an effect on the epigenetic regulation of the human placenta. We hypothesize that epigenetic deregulation of the placenta may play a role in the perinatal outcome after ART.

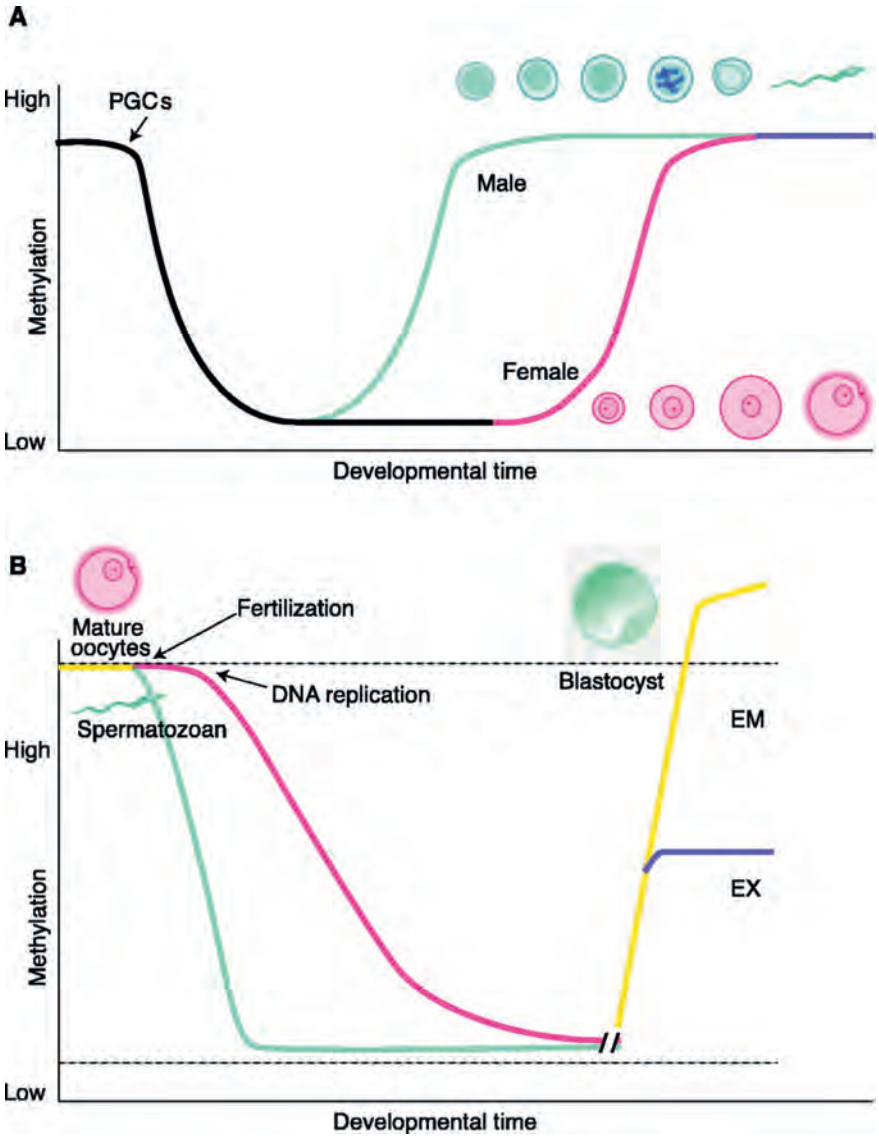


Figure 1 **(A)** Methylation reprogramming in the germ line. Primordial germ cells (PGCs) become demethylated early in development. Remethylation begins in prospermatogonia in male germ cells, and after birth in growing oocytes. **(B)** Methylation reprogramming in preimplantation embryos. The paternal genome (blue) is demethylated by an active mechanism immediately after fertilization. The maternal genome (red) is demethylated by a passive mechanism. Both are remethylated around the time of implantation to different extents in embryonic (EM) and extraembryonic (EX) lineages. Methylated imprinted genes and some repeat sequences (dashed line) do not become demethylated. Unmethylated imprinted genes (dashed line) do not become methylated. Figure from Reik *et al.* (2001).

Aims and outline

This thesis focuses on the effects of ART in humans on adverse perinatal outcome. We wished to gain more insight into its etiology and find ways to improve the safety of ART in the future. The first aim of the thesis is to investigate whether *in vitro* culture of human embryos during the first few days of preimplantation development affects perinatal outcome. In **Chapter 2,** we evaluate the effect of *in vitro* culture medium on human perinatal outcome by comparing these outcomes in singleton pregnancies after usage of two commercially available sequential media systems. In addition, the influence of various culture media on perinatal outcome in twin pregnancies as well as pregnancies after transfer of frozen embryos will be studied. In **Chapter 3**, the impact of embryo culture media on fetal growth patterns, with particular focus on the onset of growth divergence, is investigated, as abnormal fetal growth patterns are a major risk factor for the development of chronic diseases in later life.

The second aim of the thesis is to evaluate whether ART has an effect on the epigenetic regulation of the human placenta. **Chapter 4** reviews the knowledge in the field of epigenetics in relation to placental development and function. In **Chapter 5**, we investigate the possible effect of ART on the epigenetic regulation of human placentas. The DNA methylation level of several differentially methylated regions (DMRs) involved in parent-of-origin specific expression of imprinted genes, will be assessed in placentas from pregnancies conceived by ART and compared to placentas from spontaneous conception. In case of differential methylation between the two groups, the effect on gene expression will be assessed. To further explore the effect of ART on the epigenetic regulation of the human placenta, in **Chapter 6** we carry out a micro-array analysis to investigate the pathways that are deregulated, we analyse the gene expression of several placental growth-related imprinted genes and we perform a quantitative allele-specific expression analysis of the differentially expressed imprinted genes to investigate whether this differential expression is due to loss of imprinting (LOI).

References

Camargo LS, Freitas C, de Sa WF, de Moraes Ferreira A, Serapiao RV,Viana JH. Gestation length, birth weight and offspring gender ratio of in vitro-produced Gyr (Bos indicus) cattle embryos. *Anim Reprod Sci* 2010;**120**:(1-4): 10-15.

Doherty AS, Mann MR, Tremblay KD, Bartolomei MS,Schultz RM. Differential effects of culture on imprinted H19 expression in the preimplantation mouse embryo. *Biol Reprod* 2000;**62**:(6): 1526-1535.

Fauque P, Jouannet P, Lesaffre C, Ripoche MA, Dandolo L, Vaiman D,Jammes H. Assisted Reproductive Technology affects developmental kinetics, H19 Imprinting Control Region methylation and H19 gene expression in individual mouse embryos. *BMC Dev Biol* 2007;**7**: 116.

Fauque P, Mondon F, Letourneur F, Ripoche MA, Journot L, Barbaux S, Dandolo L, Patrat C, Wolf JP, Jouannet P*, et al.* In vitro fertilization and embryo culture strongly impact the placental transcriptome in the mouse model. *PLoS One* 2010;**5**:(2): e9218.

Feil D, Lane M, Roberts CT, Kelley RL, Edwards LJ, Thompson JG,Kind KL. Effect of culturing mouse embryos under different oxygen concentrations on subsequent fetal and placental development. *J Physiol* 2006;**572**:(Pt 1): 87-96.

Fernandez-Gonzalez R, de Dios Hourcade J, Lopez-Vidriero I, Benguria A, De Fonseca FR,Gutierrez-Adan A. Analysis of gene transcription alterations at the blastocyst stage related to the long-term consequences of in vitro culture in mice. *Reproduction* 2009;**137**:(2): 271-283.

Ferraretti AP, Goossens V, Kupka M, Bhattacharya S, de Mouzon J, Castilla JA, Erb K, Korsak V,Nyboe Andersen A. Assisted reproductive technology in Europe, 2009: results generated from European registers by ESHRE. *Hum Reprod* 2013.

Fraga MF, Ballestar E, Paz MF, Ropero S, Setien F, Ballestar ML, Heine-Suner D, Cigudosa JC, Urioste M, Benitez J*, et al.* Epigenetic differences arise during the lifetime of monozygotic twins. *Proc Natl Acad Sci U S A* 2005;**102**:(30): 10604-10609.

Frost JM,Moore GE. The importance of imprinting in the human placenta. *PLoS Genet* 2010;**6**:(7): e1001015.

Helmerhorst FM, Perquin DA, Donker D,Keirse MJ. Perinatal outcome of singletons and twins after assisted conception: a systematic review of controlled studies. *Bmj* 2004;**328**:(7434): 261.

Jackson RA, Gibson KA, Wu YW,Croughan MS. Perinatal outcomes in singletons following in vitro fertilization: a meta-analysis. *Obstet Gynecol* 2004;**103**:(3): 551-563.

James JL, Whitley GS,Cartwright JE. Pre-eclampsia: fitting together the placental, immune and cardiovascular pieces. *J Pathol* 2010;**221**:(4): 363-378.

Jirtle RL,Skinner MK. Environmental epigenomics and disease susceptibility. *Nat Rev Genet* 2007;**8**:(4): 253-262.

Kallen AJ, Finnstrom OO, Lindam AP, Nilsson EM, Nygren KG,Olausson PM. Cerebral palsy in children born after in vitro fertilization. Is the risk decreasing? *Eur J Paediatr Neurol* 2010a;**14**:(6): 526-530.

Kallen B, Finnstrom O, Lindam A, Nilsson E, Nygren KG,Otterblad Olausson P. Trends in delivery and neonatal outcome after in vitro fertilization in Sweden: data for 25 years. *Hum Reprod* 2010b;**25**:(4): 1026-1034.

Kallen B, Finnstrom O, Lindam A, Nilsson E, Nygren KG,Otterblad PO. Congenital malformations in infants born after in vitro fertilization in Sweden. *Birth Defects Res A Clin Mol Teratol* 2010c;**88**:(3): 137-143.

Khosla S, Dean W, Brown D, Reik W,Feil R. Culture of preimplantation mouse embryos affects fetal development and the expression of imprinted genes. *Biol Reprod* 2001;**64**:(3): 918-926.

Lane M,Gardner DK. Increase in postimplantation development of cultured mouse embryos by amino acids and induction of fetal retardation and exencephaly by ammonium ions. *J Reprod Fertil* 1994;**102**:(2): 305-312.

Li T, Vu TH, Ulaner GA, Littman E, Ling JQ, Chen HL, Hu JF, Behr B, Giudice L,Hoffman AR. IVF results in de novo DNA methylation and histone methylation at an Igf2-H19 imprinting epigenetic switch. *Mol Hum Reprod* 2005;**11**:(9): 631-640.

Lonergan P, Fair T, Corcoran D,Evans AC. Effect of culture environment on gene expression and developmental characteristics in IVF-derived embryos. *Theriogenology* 2006;**65**:(1): 137-152.

Mann MR, Lee SS, Doherty AS, Verona RI, Nolen LD, Schultz RM,Bartolomei MS. Selective loss of imprinting in the placenta following preimplantation development in culture. *Development* 2004;**131**:(15): 3727-3735.

Mansour R, Ishihara O, Adamson GD, Dyer S, de Mouzon J, Nygren KG, Sullivan E,Zegers-Hochschild F. International Committee for Monitoring Assisted Reproductive Technologies world report: Assisted Reproductive Technology 2006. *Hum Reprod* 2014.

Mantikou E, Youssef MA, van Wely M, van der Veen F, Al-Inany HG, Repping S,Mastenbroek S. Embryo culture media and IVF/ICSI success rates: a systematic review. *Hum Reprod Update* 2013;**19**:(3): 210-220.

Market-Velker BA, Fernandes AD,Mann MR. Side-by-side comparison of five commercial media systems in a mouse model: suboptimal in vitro culture interferes with imprint maintenance. *Biol Reprod* 2010;**83**:(6): 938-950.

McDonald SD, Han Z, Mulla S, Murphy KE, Beyene J,Ohlsson A. Preterm birth and low birth weight among in vitro fertilization singletons: a systematic review and meta-analyses. *Eur J Obstet Gynecol Reprod Biol* 2009;**146**:(2): 138-148.

McGovern PG, Llorens AJ, Skurnick JH, Weiss G,Goldsmith LT. Increased risk of preterm birth in singleton pregnancies resulting from in vitro fertilization-embryo transfer or gamete intrafallopian transfer: a meta-analysis. *Fertil Steril* 2004;**82**:(6): 1514-1520.

Mouillet JF, Chu T, Hubel CA, Nelson DM, Parks WT,Sadovsky Y. The levels of hypoxia-regulated microRNAs in plasma of pregnant women with fetal growth restriction. *Placenta* 2010;**31**:(9): 781-784.

Numabe T, Oikawa T, Kikuchi T,Horiuchi T. Birth weight and birth rate of heavy calves conceived by transfer of in vitro or in vivo produced bovine embryos. *Anim Reprod Sci* 2000;**64**:(1-2): 13-20.

Pandey S, Shetty A, Hamilton M, Bhattacharya S,Maheshwari A. Obstetric and perinatal outcomes in singleton pregnancies resulting from IVF/ICSI: a systematic review and meta-analysis. *Hum Reprod Update* 2012;**18**:(5): 485-503.

Pinborg A, Wennerholm UB, Romundstad LB, Loft A, Aittomaki K, Soderstrom-Anttila V, Nygren KG, Hazekamp J,Bergh C. Why do singletons conceived after assisted reproduction technology have adverse perinatal outcome? Systematic review and meta-analysis. *Hum Reprod Update* 2013;**19**:(2): 87-104.

Reik W, Dean W,Walter J. Epigenetic reprogramming in mammalian development. *Science* 2001;**293**:(5532): 1089-1093.

Rivera RM, Stein P, Weaver JR, Mager J, Schultz RM,Bartolomei MS. Manipulations of mouse embryos prior to implantation result in aberrant expression of imprinted genes on day 9.5 of development. *Hum Mol Genet* 2008;**17**:(1): 1-14.

Rooke JA, McEvoy TG, Ashworth CJ, Robinson JJ, Wilmut I, Young LE,Sinclair KD. Ovine fetal development is more sensitive to perturbation by the presence of serum in embryo culture before rather than after compaction. *Theriogenology* 2007;**67**:(3): 639-647.

Sasaki H, Ferguson-Smith AC, Shum AS, Barton SC,Surani MA. Temporal and spatial regulation of H19 imprinting in normal and uniparental mouse embryos. *Development* 1995;**121**:(12): 4195-4202.

Sazonova A, Kallen K, Thurin-Kjellberg A, Wennerholm UB,Bergh C. Factors affecting obstetric outcome of singletons born after IVF. *Hum Reprod* 2011;**26**:(10): 2878-2886.

Schwarzer C, Esteves TC, Arauzo-Bravo MJ, Le Gac S, Nordhoff V, Schlatt S,Boiani M. ART culture conditions change the probability of mouse embryo gestation through defined cellular and molecular responses. *Hum Reprod* 2012;**27**:(9): 2627-2640.

Sinclair KD, Young LE, Wilmut I,McEvoy TG. In-utero overgrowth in ruminants following embryo culture: lessons from mice and a warning to men. *Hum Reprod* 2000;**15 Suppl 5**: 68-86.

Steegers EA, von Dadelszen P, Duvekot JJ,Pijnenborg R. Pre-eclampsia. *Lancet* 2010;**376**:(9741): 631-644.

Thompson JG, Gardner DK, Pugh PA, McMillan WH,Tervit HR. Lamb birth weight is affected by culture system utilized during in vitro pre-elongation development of ovine embryos. *Biol Reprod* 1995;**53**:(6): 1385-1391.

van Montfoort AP, Hanssen LL, de Sutter P, Viville S, Geraedts JP,de Boer P. Assisted reproduction treatment and epigenetic inheritance. *Hum Reprod Update* 2012;**18**:(2): 171-197.

Young LE, Fernandes K, McEvoy TG, Butterwith SC, Gutierrez CG, Carolan C, Broadbent PJ, Robinson JJ, Wilmut I,Sinclair KD. Epigenetic change in IGF2R is associated with fetal overgrowth after sheep embryo culture. *Nat Genet* 2001;**27**:(2): 153-154.

Young LE, Sinclair KD,Wilmut I. Large offspring syndrome in cattle and sheep. *Rev Reprod* 1998;**3**:(3): 155-163.

Zander-Fox DL, Mitchell M, Thompson JG,Lane M. Alterations in mouse embryo intracellular pH by DMO during culture impair implantation and fetal growth. *Reprod Biomed Online* 2010;**21**:(2): 219-229.

Zander DL, Thompson JG,Lane M. Perturbations in mouse embryo development and viability caused by ammonium are more severe after exposure at the cleavage stages. *Biol Reprod* 2006;**74**:(2): 288-294.

Part I

Clinical observations

Chapter 2

Further evidence that culture media affect perinatal outcome: findings after transfer of fresh and cryopreserved embryos.

Nelissen ECM, Van Montfoort APA, Coonen E, Derhaag JG, Geraedts JPM, Smits LJM, Land JA, Evers JLH, Dumoulin JCM

Human Reproduction. 2012; 27(7):1966-76.

Abstract

Background: We have previously shown that the medium used for culturing IVF embryos affects the birthweight of the resulting newborns. This observation with potentially far-reaching clinical consequences during later life, was made in singletons conceived during the first IVF treatment cycle after the transfer of fresh embryos. In the present study, we hypothesize that *in vitro* culture of embryos during the first few days of preimplantation development affects perinatal outcome, not only in singletons conceived in all rank order cycles but also in twins and in children born after transfer of frozen embryos. Furthermore, we investigated the effect of culture medium on gestational age (GA) at birth.

Methods: Oocytes and embryos from consecutive treatment cycles were alternately assigned to culture in either medium from Vitrolife or from Cook. Data on a cohort of 294 live born singletons conceived after fresh transfer during any of a patient's IVF treatment cycles, as well as data of 67 singletons conceived after frozen embryo transfer (FET) and of 88 children of 44 twin pregnancies after fresh transfer were analysed by means of multiple linear regression.

Results: *In vitro* culture in medium from Cook resulted in singletons after fresh transfer with a lower mean birthweight (adjusted mean difference, 112 grams, $P = 0.03$), and in more singletons with low birthweight (LBW) <2500g ($P = 0.006$) and LBW for GA ≥ 37 weeks ($P = 0.015$), when compared with singletons born after culture in medium from Vitrolife AB. GA at birth was not related to the medium used (adjusted difference, 0.05 weeks, $P = 0.83$). Among twins in the Cook group, higher inter-twin mean birthweight disparity and birthweight discordance were found. Z-scores after FET were -0.04 (± 0.14) in the Cook group compared with 0.18 (± 0.21) in the Vitrolife group ($P > 0.05$).

Conclusions: Our findings support our hypothesis that culture medium influences perinatal outcome of IVF singletons and twins. A similar trend is seen in case of singletons born after FET. GA was not affected by culture medium. These results indicate that *in vitro* culture might be an important factor explaining the poorer perinatal outcome after assisted reproduction technology (ART). Further research is needed to confirm this culture medium-induced effect in humans and to provide more insight into whether it is caused by epigenetic disturbance of imprinted genes in fetal or placental tissues. Moreover, embryo culture media and their effects need to be investigated thoroughly to select the best embryo culture medium in order to minimize or prevent short-term risks and maybe even long-term disease susceptibility.

Introduction

The first live born after IVF, frozen embryo transfer (FET) and ICSI were reported in 1978, 1984 and 1992, respectively (Steptoe *et al.*, 1978; Zeilmaker *et al.*, 1984; Palermo *et al.*, 1992). Ever since, the use of assisted reproduction technologies (ARTs) has risen substantially. In 2006, <30 years after the birth of the first IVF child, the European IVF-monitoring Consortium reported 117 318 treatment cycles with IVF, 232 844 treatment cycles with ICSI and 86 059 FETs (de Mouzon *et al.*, 2010). In 2006, 1.0 - 4.1% of all newborns in Europe were associated with *in vitro* techniques (de Mouzon *et al.*, 2010). Therefore, it is important to monitor the consequences of handling gametes and embryos during ART. IVF singletons, when compared with naturally conceived singletons, are at significantly increased risk of low birthweight (LBW), preterm birth, small for gestational age (SGA), perinatal mortality (Jackson *et al.*, 2004; McDonald *et al.*, 2009) and birth defects (Rimm *et al.*, 2004; Hansen *et al.*, 2005). LBW is associated with chronic diseases expressed later in life, e.g. cardiovascular disease, hypertension and type 2 diabetes (Barker, 2004), while preterm birth is associated with increased morbidity, mortality and diminished long-term survival and reproduction (McIntire *et al.*, 2008; Swamy *et al.*, 2008). Moreover, dizygotic IVF twins have a worse perinatal outcome when compared with naturally conceived dizygotic twins, such as increased risks of LBW and preterm birth (Hansen *et al.*, 2009; Kallen *et al.*, 2010; McDonald *et al.*, 2010b). However, some studies did not find an unfavourable outcome in IVF twins when they restricted their analyses to dizygotic pairs (Pinborg *et al.*, 2004; Boulet *et al.*, 2008). When children born after FET are compared with those born after fresh embryo transfer, the perinatal outcome after FET appears to be similar or even improved, especially regarding LBW and preterm birth (Wennerholm *et al.*, 2009; Pelkonen *et al.*, 2010; Pinborg *et al.*, 2010). However, the outcome after FET seems poorer when compared with naturally conceived singletons (Pelkonen *et al.*, 2010; Pinborg *et al.*, 2010). The cause of these unfavourable pregnancy and neonatal outcomes might be attributed to one or more aspects of the reproductive technology itself (hormonal stimulation, *in vitro* culture and embryo transfer) or to patient-related factors (Thomson *et al.*, 2005; De Geyter *et al.*, 2006; Ombelet *et al.*, 2006; Romundstad *et al.*, 2008; Pelinck *et al.*, 2010). Parameters of ovarian stimulation as part of an IVF treatment (duration of stimulation, consumption of gonadotrophins and number of oocytes retrieved) have been studied and appear not to be associated with birthweight (Griesinger

et al., 2008). Recently, we have shown that in IVF cycles with transfer of fresh embryos, the culture medium used during the first 2 - 3 days affects birthweight (Dumoulin *et al.*, 2010). In the patients' initial IVF treatment cycles, we found that Vitrolife culture medium was significantly associated with increased birthweight when compared with Cook medium (Dumoulin *et al.*, 2010). This remarkable finding with potentially far-reaching consequences needs to be confirmed in a larger prospective intervention study. Therefore, a multi-centre trial has been initiated recently. Meanwhile, we investigated this finding in a larger cohort and other readily available groups. The aim of our present study was to investigate in a larger cohort whether the same culture medium-dependent effect on birthweight and possibly also on GA, occurs in live born singletons after transfer of fresh embryos, irrespective of the IVF treatment cycle rank order. This could be a confounder since patients not conceiving in their first treatment cycle might consist of a selected group of patients with different characteristics when compared with couples who do conceive. In addition, the influence of different culture media on perinatal outcome of twin pregnancies as well as pregnancies after transfer of frozen embryos was studied. Our hypothesis is that *in vitro* culture of embryos during the first few days of preimplantation development affects perinatal outcome, not only in singletons after transfer of fresh embryos but also in twins and in children born after transfer of frozen embryos.

Materials and methods

Study design

In the present prospective cohort study at the Maastricht University Medical Centre (MUMC), all treatment cycles from couples undergoing IVF or ICSI from July 2003 to December 2006 were assigned alternately to culture media from Vitrolife AB (Göteborg, Sweden) or Cook (Brisbane, Australia). These two sequential culture media were commercially available and both are widely used. By strictly alternating, treatment allocation to one of the two media was performed on the day preceding the ovum pick up by laboratory technicians unaware of patient characteristics. In contrast, the order of ovum pick-ups on a certain day, which directly determined the allocation to a study group, was planned by clinical personnel unaware of the laboratory allocation procedure. This alternating allocation to one of the two media was part of our internal

quality monitoring system in order to be able to identify suboptimal batches of a particular medium. In the present analysis, results of all IVF treatment cycles meeting the inclusion criteria and resulting in a live born child were included. Data analysis was performed on the outcome of all singleton pregnancies resulting from both fresh and cryopreserved embryo transfers as well as twin pregnancies resulting from fresh embryo transfers. All participating couples gave written informed consent for the use of their data. The local medical ethics committee approved the collection of data for quality monitoring purposes as part of our IVF treatment protocol.

Study population and data collection

During the study period, with the exception of a few cases, only patients of $\leq$40 years of age and with a BMI of <30 kg/m^2, were admitted to our IVF program and included in the study. Patients applying for preimplantation genetic diagnosis or requiring donor oocytes were excluded from the study. Smoking habits and parental weight and height were recorded at the start of each IVF treatment cycle. Only data from singletons and twins born alive after the 20th week of gestation were included in the data analysis. Furthermore, data from only one singleton or pair of twins per couple was used in the data analysis. Thus, each couple could contribute only one pregnancy to this study. Pregnancies lost to follow-up were excluded from data analysis. After delivery, the obstetricians or midwives were contacted to obtain information about complications during pregnancy (gestational diabetes, hypertension and pre-eclampsia) and perinatal outcomes (birthweight, GA and gender).

IVF procedures

Except for the media, exactly the same ovarian stimulation, fertilization, culture and embryo transfer procedures were applied in both groups. Detailed IVF procedures and embryo culture procedures have been described earlier (Dumoulin *et al.*, 2010). Briefly, patients received long down-regulation with GnRH agonists and underwent ovarian stimulation with recombinant FSH. Thirty-six hours after HCG injection, ultrasound-guided oocyte retrieval was carried out and luteal support consisted of vaginally administered progesterone. In case of a pregnancy, progesterone was continued until 7 weeks of gestation. The day before oocyte retrieval, dishes containing droplets of culture medium were prepared. In case of IVF, oocytes were inseminated in either IVF-50^{TM}

(Vitrolife, Göteborg, Sweden) or K-SIFM (Cook, Brisbane, Australia) medium. After checking for the presence of pronuclei, zygotes were subsequently transferred to either G1™ Version 3 (Vitrolife group) or K-SICM (Cook group) medium and cultured individually. In case of ICSI, oocytes were individually cultured in either G1™ Version 3 (Vitrolife group) or K-SICM (Cook group) medium immediately after injection. All media from both suppliers were ready-to-use and included 5 mg/ml pharmaceutical grade human serum albumin (HSA). Culture conditions were 37ºC, 5% O_2, 6% CO_2 and 89% N_2. Embryo transfer was performed under ultrasound-guidance on Day 2 or Day 3 after oocyte retrieval. A single embryo was transferred when female age was <38 years and at least one good quality embryo was available. In the remaining cases, two embryos were transferred. Cryopreservation of supernumerary embryos was performed on Day 3 after oocyte retrieval according to a conventional slow freezing protocol with dimethylsulfoxide (Van der Elst *et al.*, 1995). Only embryos that had reached the 8-cell stage and with a sufficient morphological quality, were cryopreserved. Embryos were transferred in an artificial cycle created with orally administered estradiol (E_2) and vaginal progesterone. After thawing on the day of the transfer, two embryos, if available, were transferred. In case of a pregnancy, E_2 and progesterone were continued until 12 weeks of gestation.

Definitions

GA was calculated from the day of oocyte retrieval which was defined as Day 14 of the cycle. GA in a frozen cycle was calculated from the day of embryo transfer which was defined as Day 17 (due to cryopreservation on Day 3). Clinical pregnancy was defined as the presence of fetal heart activity on ultrasound performed 5–6 weeks after embryo transfer. Preterm birth and very preterm birth were defined as delivery before 37 and 32 completed weeks of gestation, respectively. LBW, very low birthweight (VLBW) and high birthweight (HBW) were defined as birthweight <2500 g, <1500 g and >4500 g, respectively. SGA was defined as birthweight below the 10th percentile for GA, whereas very SGA infants were defined as below the 3rd percentile. Large for GA (LGA) and very LGA infants were defined as above the 90th percentile and 97th percentile, respectively (Oken *et al.*, 2003). In case of a twin pregnancy, the inter-twin birthweight disparity was calculated in grams and as a percentage. The inter-twin percentage birthweight disparity was calculated by the difference in birthweight divided by the weight of the larger twin and then multiplied by 100. Birthweight discordance of twins

was defined as >25% difference in birthweight of the two children (Blickstein *et al.*, 2003). Congenital defects were classified as major malformations when they caused functional impairment or required surgical correction. The remaining congenital defects were considered minor (Bonduelle *et al.*, 2002).

Statistical analysis

Crude differences of the primary (birthweight and GA) and the secondary outcomes (SGA, LGA, congenital defects, birthweight disparity in case of twins) between both study groups were tested by use of the Student's t-test in the case of continuous variables. For binary variables, we used the χ^2-test or in case of sample sizes lower than five observations per cell the Fisher's exact test. When appropriate, Yates' correction was applied. Two-sided $P < 0.05$ were considered to indicate statistical significance. A relative risk was calculated to compare the rate of events occurring at any given point in time. In case of two quantitative discrete variables, the relative risk could be calculated, however, not in case of continuous variables or zero numbers. To compare singleton children born at different GAs and from different genders, a z-score (weight of individual child minus median weight of a reference population of children born at the same GA and of the same gender divided by standard deviation) was assigned to each child (Oken *et al.*, 2003; Land, 2006). In singletons, the associations of type of culture medium with birthweight and GA at delivery were further analysed by use of multiple linear regression, while controlling for the following covariates: fertilization method (IVF or ICSI), maternal and paternal height, weight, smoking and age, gender, number of transferred embryos, day of embryo transfer (second or third), primary or secondary subfertility, treatment cycle rank order, pregnancy complications (gestational diabetes, hypertension and pre-eclampsia), cause of subfertility and duration of subfertility. Where birthweight was the outcome, we additionally controlled for GA at delivery. β is the regression coefficient. These regression coefficients are the estimates resulting from an analysis carried out on variables that have been standardized (so that their variances are 1) to be able to investigate the effect of independent variables which are measured in different units of measurement (e.g. maternal height in cm and duration of subfertility in years). We performed checks of normality of the distributions of residuals and of homogeneity of the variance (homoscedasticity) by means of the Shapiro-Wilk test and White test. No Bonferroni adjustments were applied for multiple testing (Rothman *et al.*, 2008).

Results

Patient and cycle characteristics

A total of 1432 IVF treatment cycles of which 715 were allocated to culture in Vitrolife medium and 717 allocated to culture in Cook medium were performed during the study period and resulted in 210 and 168 clinical pregnancies after fresh embryo transfer respectively (Figure I). Pregnancy losses before the 20th week of gestation (10 in Vitrolife and 10 in Cook) and stillborn children after 20 weeks of gestation (two in Vitrolife and three in Cook) were excluded. A few couples had more than one delivery during the study period. These additional deliveries were excluded (nine in Vitrolife versus three in Cook group). Thus, each couple contributed only one pregnancy to this study. The outcome of one pregnancy after transfer of fresh embryos in the Cook group was unknown. This resulted in 189 live births in the Vitrolife group versus 151 in the Cook group. Despite transfer of maximum two embryos, we recorded two triplet pregnancies (one in each study group), which were excluded from all analyses as well. The remaining pregnancies (188 in Vitrolife versus 150 in Cook) resulted in a total of 168 live born singletons and 20 twins in the Vitrolife group versus 126 singletons and 24 twins in the Cook group.

Of the 294 live born singletons, 188 children were born after the first IVF treatment cycle. These data have already been published (Dumoulin *et al.*, 2010). In the present study, we further analysed the total cohort of 294 children conceived in all rank order cycles and the smaller subgroup of 106 children born after fresh embryo transfer in treatment cycles 2, 3 and 4 (58 in the Vitrolife group and 48 in the Cook group). Parent and cycle characteristics are shown in Table I. Parental height and weight were slightly higher in the Vitrolife group. There were more women smoking ≥10 cigarettes per day in the Vitrolife group, while more men were smoking ≥10 cigarettes per day in the Cook group. The rate of single embryo transfer (SET) was higher in the Cook group.

In the 44 twin pregnancies, no differences in maternal and paternal age, height and weight and duration of subfertility were found between the two groups (data not shown).

Transfer of thawed embryos frozen during the study period, resulted in 67 singleton live borns (22 in Vitrolife versus 45 in Cook). Couples could participate only once in our study, which means that all these 67 couples had not conceived after fresh embryo transfer. In contrast with the fresh embryo

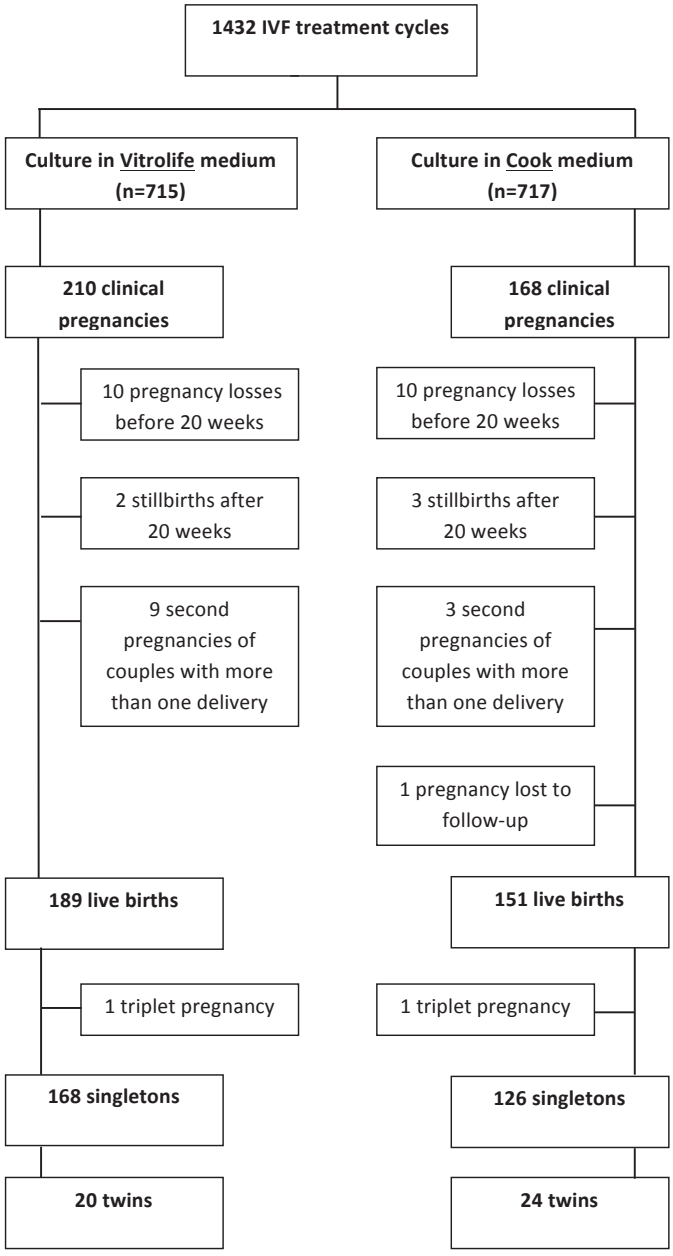


Figure I Flow chart of the study (fresh embryo transfer).

transfer cycle characteristics, in FET cycles paternal height and weight were slightly higher in the Cook group compared with the Vitrolife group (Table II).

Table I Parental and cycle characteristics of singletons born after fresh embryo transfer in treatment cycles > 1 (2 - 4) and in all cycles.

Characteristic	Cycle > 1		All cycles	
	Vitrolife group (n=58)	Cook group (n=48)	Vitrolife group (n=168)	Cook group (n= 126)
Primary indication for IVF treatment				
Tubal factor	8 (13.8)	4 (8.3)	18 (10.7)	17 (13.5)
Male factor	36 (62.1)	32 (66.7)	107 (63.7)	80 (63.5)
Unexplained	12 (12.1)	11 (22.9)	36 (21.4)	25 (19.8)
Other	2 (3.4)	1 (2.1)	7 (4.2)	4 (3.2)
Duration of subfertility (years)	4.0 ± 1.8	3.7 ± 2.1	3.4 ± 1.7	3.5 ± 1.9
Primary subfertility	46 (79.3)	33 (68.8)	127 (75.6)	92 (73.0)
Cycles with ICSI	36 (62.1)	33 (68.8)	109 (64.9)	81 (64.3)
Cycles with transfer on Day 2	44 (75.9)	36 (75.0)	130 (77.4)	95 (75.4)
Cycles with transfer on Day 3	14 (24.1)	12 (25.0)	38 (22.6)	31 (24.6)
Single embryo transfer (SET)	28 (48.3)	26 (54.2)	81 (48.2)	75 (59.5)
Maternal characteristics				
Age (years)	33.0 ± 4.1	33.4 ± 3.7	32.4 ± 4.0	32.6 ± 3.6
Age ≥ 38 years	8 (13.8)	5 (10.4)	13 (7.7)	8 (6.4)
Height (cm)	168.0 ± 6.1	166.9 ± 7.2	169.4 ± 5.8	167.6 ± 7.4
Weight (kg)	69.8 ± 9.5	67.9 ± 10.4	70.1 ± 10.5	67.1 ± 10.1
Body mass index (range)	24.7 (18.3-31.9)	24.3 (18.1-31.2)	24.4 (17.5 – 33.3)	23.9 (18.0 – 31.4)
Smoking ≥ 10 cigarettes/day	10 (17.2)	5 (10.4)	26 (15.5)	17 (13.5)
Paternal characteristics				
Age (years)	35.4 ± 5.9	35.4 ± 3.9	35.4 ± 5.7	35.8 ± 5.0
Height (cm)	181.9 ± 7.7	180.7 ± 7.7	182.1 ± 7.8	180.9 ± 7.4
Weight (kg)	87.2 ± 12.8	84.3 ± 9.9	86.4 ± 13.9	83.1 ± 11.1
Body mass index (range)	26.3 (17.9-37.5)	25.8 (20.8-27.2)	26.0 (17.9 – 46.0)	25.4 (19.9 – 34.3)
Smoking ≥ 10 cigarettes/day	9 (15.5)	7 (14.6)	29 (17.3)	25 (19.8)

Data are presented as numbers (%) or mean ± SD.

Pregnancies

The occurrence of pregnancy complications (gestational diabetes, hypertension and pre-eclampsia), was similar in both groups after fresh embryo transfer [14 (8.3%) and 2 (10.0%) in the Vitrolife group versus 9 (7.1%) and 1 (4.2%) in the Cook group for singleton and in twin pregnancies, respectively] as well as after FET [1 (4.6%) in the Vitrolife group and 2 (4.4%) in the Cook group].

The number of vanishing twins after fresh embryo transfer was two in the Vitrolife group and one in the Cook Group. After FET, there was one vanishing twin in the Vitrolife group and three in the Cook group.

Table II Parental characteristics of singletons born after frozen embryo transfer.

Characteristic	Vitrolife group (n=22)	Cook group (n= 45)
Maternal characteristics		
Age (years)	33.2 ± 3.8	33.9 ± 3.3
Height (cm)	167.9 ± 7.8	168.4 ± 6.7
Weight (kg)	68.8 ± 11.8	68.8 ± 11.5
Paternal characteristics		
Age (years)	36.6 ± 5.7	36.9 ± 4.3
Height (cm)	179.5 ± 5.8	184.9 ± 6.9
Weight (kg)	86.8 ± 16.0	90.3 ± 12.8
Duration of subfertility (years)	3.1 ± 1.4	3.8 ± 2.7

Data are mean ± SD.

Perinatal outcome

Singletons

Perinatal outcome of all singletons after fresh transfer in our study cohort, 168 in the Vitrolife group and 126 in the Cook group, is presented in Table III. Mean difference in birthweight between Vitrolife and Cook was 183 g. Mean birthweight as well as mean birthweight adjusted for GA and gender (z-score), were different between the study groups when all cycles were analysed (P = 0.006 and P = 0.007). The shift in birthweight distribution between the two media is visualised in Figure II. Furthermore, there were significantly more singletons with LBW (P = 0.006) and also with LBW for GA ≥37 weeks (P = 0.015) in the Cook group. Even in the smaller subgroup of IVF treatment cycle >1 (cycles 2 - 4), the frequency of LBW was statistically significantly different between the study groups (P = 0.029). On the other hand, although statistically not significant, five children in the Vitrolife group had a HBW compared with no children in the Cook group. The rate of children with congenital defects was not different between groups. Major malformations were seen in 6 singletons (2 in the Vitrolife group and 4 in the Cook group), whereas minor malformations were seen in 15 singletons (8 in Vitrolife and 7 in Cook).

Table III Neonatal outcome of live born singletons after fresh embryo transfer.

Characteristic	Cycle > 1		All cycles			*P*-value
	Vitrolife group (n=58)	Cook group (n=48)	Vitrolife group (n=168)	Cook group (n= 126)	Relative risk (95% CI)	
Boys	32 (55.2)	24 (50.0)	81 (48.2)	59 (46.8)	1.03 (0.81-1.32)	NS (a, b)
Gestational age (GA) at birth	39.6 ± 0.2	39.4 ± 0.3	39.6 ± 0.1	39.4 ± 0.2		NS
Preterm birth (<37 weeks)	1 (1.7)	3 (6.3)	6 (3.6)	8 (6.4)	0.56 (0.21-1.52)	NS
Very preterm birth (<32 weeks)	0	1 (2.1)	1 (0.6)	3 (2.4)	0.25 (0.04-1.73)	NS
Birthweight (g)	3384 ± 76	3322 ± 87	3436 ± 44	3253 ± 50		a=NS, b=0.006
Z-score	-0.14 ± 0.15	-0.14 ± 0.15	0.05 ± 0.08	-0.265 ± 0.08		a=NS, b=0.007
Low birthweight (<2500g)	1 (1.7)	6 (12.5)	4 (2.4)	12 (9.5)	0.25 (0.09-0.72)	a=0.029, b=0.006
Low birthweight with GA ≥ 37 weeks	1 (1.7)	4 (8.3)	2 (1.2)	8 (6.4)	0.19 (0.05-0.76)	a=NS, b=0.015
Very low birthweight (<1500g)	0	1 (2.1)	2 (1.2)	3 (2.4)	0.50 (0.10-2.48)	NS
High birthweight (>4500g)	1 (1.7)	0	5 (3.0)	0		NS
Small for GA (<10th perc.)	11 (19.0)	7 (14.6)	16 (9.5)	16 (12.7)	0.75 (0.39-1.43)	NS
Very small for GA (<3rd perc.)	2 (3.5)	3 (6.3)	4 (2.4)	8 (6.4)	0.38 (0.12-1.15)	NS
Large for GA (>90th perc.)	6 (10.3)	3 (6.3)	14 (8.3)	5 (4)	2.10 (0.81-5.53)	NS
Very large for GA (>97th perc.)	2 (3.5)	0	7 (4.2)	1 (0.8)	5.25 (0.86-32.65)	NS

Data are presented as numbers (%) or mean ± SEM. NS, not significant. Z-score (weight of individual child minus median weight of a reference population of children born at the same GA and of the same gender divided by standard deviation).
* *P*-value in a: cycle>1 and b: all IVF treatment cycles.

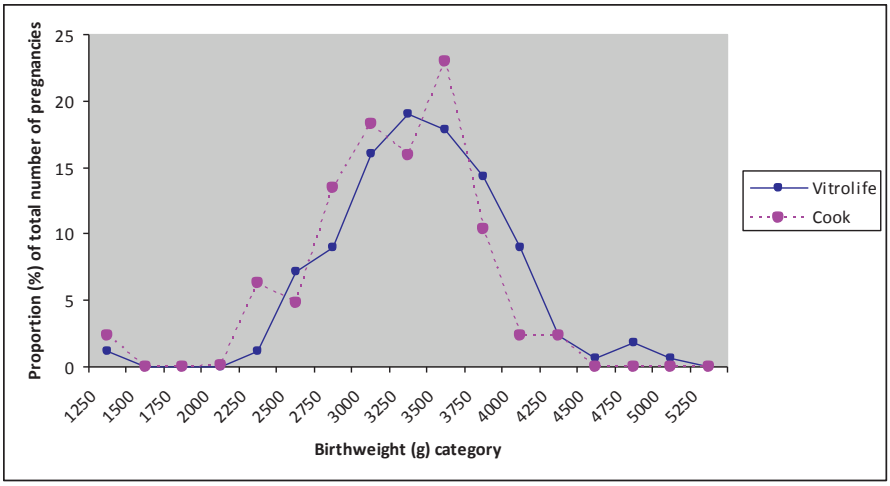


Figure II Birthweight distributions of live born singletons after fresh embryo transfers resulting from embryo culture in either Vitrolife or Cook sequential media.

We performed a multiple linear regression analysis with parity, pregnancy complications, GA, characteristics of both parents (age, weight, height and smoking habits), duration of subfertility, cause of subfertility, fertilization method (standard IVF or ICSI), cycle rank order, day of embryo transfer (second or third), number of transferred embryos (one or two), culture medium and gender as possible covariates (Table IV).

Adjusted birthweight difference was 112 g in favour of the Vitrolife medium [95% confidence interval (CI): 11 - 214 g, $P = 0.03$]. As pregnancy complications may theoretically be on the causal pathway between type of medium and lower birthweight, we also performed an analysis without this factor. The results were very similar (change of 0.1 g). The Shapiro-Wilk test and White test indicated that residuals were distributed normally and that there was no important heteroscedasticity (*P*-values 0.12 and 0.76, respectively). Besides the type of culture medium, GA ($P < 0.0001$), gender ($P < 0.001$), parity ($P < 0.001$) and height of the mother ($P = 0.008$) were significantly associated with birthweight. Mean difference in GA between Vitrolife and Cook was 0.15 weeks. Multiple linear regression with GA at birth as an outcome indicated that there was no important difference between the two culture media (adjusted difference 0.05 weeks, 95% CI -0.38 to 0.48 weeks) (Table IV). GA appeared to be positively related to maternal weight (per kg) (adjusted difference 0.03 weeks, 95% CI 0.01 - 0.05 weeks, $P = 0.02$). Again, results were similar with or without

Table IV Results of multiple regression analysis among live born singletons after transfer of fresh embryos.

Variable	Birthweight (grams)			Gestational age at birth (weeks)		
	Adjusted			Adjusted		
	β[a]	95%-CI	*P*-value	β	95%-CI	*P*-value
Vitrolife (versus Cook)	112	11 to 214	0.031	0.05	-0.38 to 0.48	NS
ICSI (versus IVF)	-2	-214 to 210	NS	-0.30	-1.19 to 0.60	NS
Maternal height (per cm)	12	3 to 21	0.008	-0.02	-0.06 to 0.02	NS
Maternal weight (per kg)	5	0 to 11	NS	0.03	0.01 to 0.05	0.016
Paternal height (per cm)	3	-5 to 11	NS	0.02	-0.01 to 0.05	NS
Paternal weight (per kg)	3	-2 to 8	NS	0.00	-0.02 to 0.02	NS
Maternal smoking (per cig.)	-1	-10 to 8	NS	0.02	-0.01 to 0.06	NS
Paternal smoking (per cig.)	0	-9 to 8	NS	-0.06	-0.09 to -0.02	<.001
Maternal age (per year)	-15	-31 to 2	NS	-0.02	-0.09 to 0.05	NS
Paternal age (per year)	10	-1 to 21	NS	-0.03	-0.08 to 0.02	NS
Secondary subfertility (versus primary)	220	95 to 345	<.001	0.06	-0.47 to 0.59	NS
Duration of subfertility (per year)	5	-26 to 36	NS	0.02	-0.11 to 0.15	NS
Cause of subfertility						
Male versus other	-198	-479 to 81	NS	0.23	-0.95 to 1.41	NS
Unexplained versus other	-165	-469 to 139	NS	0.32	-0.96 to 1.61	NS
Tubal versus other	-32	-349 to 285	NS	-0.01	-1.34 to 1.33	NS
Gestational age at birth (per week)	171	142 to 199	<.001			
Child's gender (male versus female)	177	73 to 281	<.001	0.07	-0.37 to 0.51	NS
No. of transferred embryos (2 versus 1)	38	-63 to 139	NS	-0.09	-0.52 to 0.34	NS
Day of transfer (3 versus 2)	80	-40 to 200	NS	0.49	-0.01 to 0.99	NS
Cycle number (>1 versus 1)	13	-96 to 122	NS	-0.05	-0.51 to 0.41	NS
Pregn. complications (yes or no)	-150	-344 to 43	NS	-0.53	-1.34 to 0.29	NS

95%-CI, confidence interval; NS, not significant.

[a] β (Beta) is the regression coefficient. With birthweight as an outcome, a beta of 112 for Vitrolife versus Cook means that neonates conceived in medium from Vitrolife weighed an estimated 112 g more than neonates conceived in medium from Cook. A **β** of 12 for maternal height (per cm) means that each additional cm adds an estimated 12 g to neonatal birthweight.

inclusion of pregnancy complications among the covariates. There was no heteroscedasticity according to the White test ($P > 0.99$), but, for this outcome, residuals were not distributed normally (Shapiro-Wilk test, $P < 0.0001$). Although linear regression is usually robust to violation of the normality assumption, we performed an additional logistic regression analysis with the same covariates in order to confirm our observations. As expected, the risk of preterm birth was similar for children conceived in either medium [odds ratio (OR) 0.96, 95% CI 0.27 - 3.53 for Cook versus Vitrolife, $P > 0.05$].

Since a sex-related growth difference has been found in human blastocysts after ICSI with higher mean cell log numbers per male embryo (Dumoulin *et al.*, 2005), one might question whether gender or fertilization method interacts between culture medium and birthweight. In this *post hoc* subgroup analysis, no significant effect of gender was seen, although the birthweight of boys (β= -152 g, $P = 0.06$) seemed to be more affected by culture medium than that of girls (β= -71 g, $P = 0.32$). In a second *post hoc* subgroup analysis, we investigated the potential interaction between the fertilization method (IVF or ICSI) and the culture medium. Again, no significant interaction was observed, but after IVF the impact of the culture medium on birthweight seemed to be larger (β= -192 g, $P = 0.06$) than after ICSI (β= -38 g, $P = 0.54$). Both observations need to be investigated in larger studies.

To further evaluate the possibility that differences in preimplantation embryonic growth in both media could be a confounding factor, we performed a *post hoc* analysis on a subgroup of 113 children that were born from singleton pregnancies resulting from fresh SET at Day 2 of development. The following embryonic growth parameters were added as covariates in the multiple linear regression analysis model: cleavage stage and morphological grade (1–4, with grade 4 being the best grade). In this selected group, GA ($P < 0.001$), gender of the infant ($P = 0.047$), weight of the mother ($P = 0.044$) and type of culture medium ($P = 0.029$) were significantly associated with birthweight (multiple correlation coefficient $R = 0.689$), while all other covariates including embryonic growth parameters were not.

Twins

Among the 88 children from 44 twin pregnancies, in the Cook group mean birthweight appeared lower and the number of LBW children significantly higher than in the Vitrolife group ($P > 0.05$) (Table V). The inter-twin mean birthweight disparity was significantly higher in the Cook group compared with the Vitrolife group when calculated in grams ($P = 0.045$). In the Vitrolife group, no twins were discordant for birthweight, while in the Cook group six twins were discordant ($P = 0.019$), three twins of same-sex and three of opposite-sex. Birthweight discordance is generally more common in spontaneously conceived monozygotic twins compared with dizygotic twins (Rydhstroem, 1996). A *post hoc* subgroup analysis without the two monozygotic twins in the Vitrolife group and one in the Cook group, revealed that the number of discordant twins in

both groups did not change ($P = 0.022$). Major malformations were seen in four children (two in the Vitrolife group and two in the Cook group), whereas minor malformations were seen in three children (one in Vitrolife and two in Cook).

Table V Neonatal outcome of live born twins after fresh embryo transfer

Characteristic	Vitrolife group (n=20)	Cook group (n= 24)	Relative risk (95%-CI)	*P*-value
Monozygotic twins (n)	2 (10.0)	1 (4.2)	2.50 (0.34-18.83)	NS
Gestational age (weeks)	36.3 ± 0.4	35.5 ± 0.3		NS
Preterm birth (n) (<37)	9 (45.0)	16 (66.7)	0.68 (0.39-1.14)	NS
Very preterm birth (<32)	1 (5.0)	1 (4.2)	1.20 (0.13-11.34)	NS
Low birthweight children (<2500g)	23 (58)	31 (65)	0.89 (0.64-1.23)	NS
Very low birthweight children (<1500g)	3 (7.5)	1 (2)	3.60 (0.53-24.96)	NS
Mean birthweight (g)	2400 ± 81	2284 ± 68		NS
Mean birthweight disparity (g)	259 ± 32	465 ± 93		0.045
Mean birthweight disparity (%)	10.3% ± 1.3	15.1% ± 2.3		NS
Birthweight discordance (disparity ≥ 25%)	0 (0)	6 (25)		0.019

Data are mean ± SEM or n (%). NS, not significant.

Singletons after cryopreservation

The perinatal outcome of 67 singletons born after FET was analysed (22 in the Vitrolife group and 45 in the Cook group) (Table VI). The z-score (mean birthweight adjusted for GA and gender) was 0.18 ± 0.21 in the Vitrolife group compared with -0.04 ± 0.14 in the Cook group ($P > 0.05$). None of the other neonatal characteristics in Table II were statistically significantly different between the study groups. Major malformations were seen in two singletons after FET (both in the Vitrolife group), whereas minor malformations were seen in two singletons (one in each group).

Table VI Neonatal outcome of singletons born after FET.

Characteristic	Vitrolife group (n=22)	Cook group (n= 45)	Relative risk (95%-CI)	*P*-value
Boys	10 (45.5)	23 (51.1)	0.89 (0.50-1.45)	NS
Gestational age (GA) at birth	39.3 ± 0.3	39.4 ± 0.2		NS
Preterm birth (<37 weeks)	1	2	1.02 (0.14-7.59)	NS
Very preterm birth (<32 weeks)	0	0		
Birthweight (g)	3465 ± 107	3394 ± 77		NS
Z-score	0.18 ± 0.21	-0.04 ± 0.14		NS
Low birthweight (<2500g)	0	0		
High birthweight (>4500g)	0	2		
Small for GA (<10th percentile)	1	3	0.68 (0.10-4.54)	NS
Very small for GA (<3rd percentile	1	0		
Large for GA (>90th percentile)	1	4	0.51 (0.08-3.19)	NS
Very large for GA (>97th percentile)	0	0		

NS, not significant.
Data are presented as numbers (%) or mean ± SEM. Z-score (weight of individual child minus median weight of a reference population of children born at the same GA and of the same gender divided by standard deviation).

Discussion

The main findings of this study are that *in vitro* culture of embryos in media from Cook resulted in singletons with a lower mean birthweight (adjusted mean difference 112 g), and more singletons with a LBW (<2500g) and LBW for GA ≥ 37 weeks, when compared with singletons born after culture in medium from Vitrolife AB. These results are consistent with our previous findings, based on first IVF treatment cycles with transfer of fresh embryos (Dumoulin *et al.*, 2010). There also appears to be a trend toward LGA in the Vitrolife group, which requires further study. GA at birth was not influenced by the culture medium used, but appeared to be positively related to maternal weight. A decreased risk of spontaneous preterm birth in overweight or obese women has been described before (Hendler *et al.*, 2005), although a meta-analysis found an opposite effect (McDonald *et al.*, 2010a). The negative effect of paternal smoking on GA is difficult to interpret. Presumably, this is a false-positive finding. Although GA is an important factor for birthweight, it was unrelated to culture medium and therefore did not act as a confounder. Also, cycle rank order was no confounder.

Among twins in the Cook group, a trend towards a lower mean birthweight and higher numbers of LBW children was seen. Statistical

significance was not reached, possibly due to small numbers. Interestingly, we found a significantly higher inter-twin mean birthweight disparity and birthweight discordance among twins in the Cook group compared with the Vitrolife group. It is difficult to interpret the present finding of a higher birthweight discordance among twins in the Cook group, but fetal or placental dissimilarity perhaps induced by culture medium could be a plausible hypothesis. Birthweight discordance in twins is associated with adverse neonatal outcomes, such as LBW, VLBW, neonatal intensive care unit admission and neonatal mortality (Amaru *et al.*, 2004). There appears to be no significant difference in birthweight discordance between IVF and non-IVF dizygotic twins (Suzuki *et al.*, 2007; Kallen *et al.*, 2010), although others found a higher discordance rate among IVF twins (Koudstaal *et al.*, 2000; Pinborg *et al.*, 2004). Birthweight discordance might be caused by sex differences. However, in our results, discordance was seen in three opposite-sex and three same-sex twins, which is in agreement with the findings of Pinborg *et al*. (2004) that the risk of discordant birthweight in opposite-sex and same-sex IVF twins is similar.

When we compared the z-scores of the singletons born after FET (0.18 ± 0.21 in Vitrolife versus -0.04 ± 0.14 in Cook, $P > 0.05$) with the z-scores of those born after fresh transfer (0.05 ± 0.08 in Vitrolife versus -0.265 ± 0.08 in Cook, $P = 0.007$), the birthweight of the newborns appeared to be increased after FET in both study groups, which is in agreement with the existing literature regarding similar or even improved perinatal outcomes after FET (Wennerholm *et al.*, 2009; Pelkonen *et al.*, 2010; Pinborg *et al.*, 2010). However, also after FET, a trend towards lower z-scores was noted in the Cook group compared with the Vitrolife group. This was not statistically significant, probably due to the small sample sizes in the FET group. Because a similar differential effect of these culture media is even seen in the FET group, these findings support our hypothesis that culture medium affects perinatal outcome.

We recognize that our study has limitations with respect to the allocation procedure, being a strictly alternate-case, quasi-random one. However, as allocation was performed with two-sided allocation concealment and blinding, although not perfect, this alternate allocation to one of two media closely approaches an optimal randomisation procedure. The single-centre nature of our study limits generalizability, which has led to the initiation of a multi-centre trial.

This study enables a reliable comparison of the effects of two commercially available embryo culture media since exactly the same ovarian stimulation, fertilization, culture and embryo transfer procedures were applied in both groups. Furthermore, we were not only able to investigate the influence of embryo culture media on the perinatal outcome of pregnancies after fresh embryo transfer, but also of pregnancies after FET. Our data show a comparable, culture medium-dependent influence on fresh singletons, twins as well as a similar trend in case of FET singletons. Although the effect of the type of culture medium was not significant in all analysed subgroups, the direction of the difference always remained consistent and never displayed a reversed effect.

To our knowledge, this study and a previous one of our group (Dumoulin *et al.*, 2010) have been the first to have documented the influence of culture medium on the birthweight of IVF singletons in the human. Several animal studies have been performed to address this issue. Different culture conditions, mainly achieved by the addition of serum, have led to an increased birthweight in sheep and cattle, and a decreased birthweight in mice (Khosla *et al.*, 2001; Rooke *et al.*, 2007). An explanation for these culture medium-induced effects could be that *in vitro* culture leads to epigenetic disturbance which in turn might affect developmental programming of fetal and placental tissues. Preimplantation embryo culture has been shown to affect methylation and expression of imprinted genes in several animal models (Khosla *et al.*, 2001; Young *et al.*, 2001; Mann *et al.*, 2004; Fauque *et al.*, 2010).

Over the past decades, mammalian embryo culture conditions have improved, e.g. by optimizing the composition of chemically defined culture media, investigating the role of oxygen tension, supplementation of amino acids, addition of proteins and possibly the use of sequential media systems to mimic the dynamic *in vivo* environment of early embryo development in the oviduct (Lane *et al.*, 2007; Biggers *et al.*, 2008; Sepulveda *et al.*, 2009; Hambiliki *et al.*, 2010; Hentemann *et al.*, 2010; Wirleitner *et al.*, 2010). Furthermore, culture media containing a stable glutamine derivate and vitamins or the use of HSA and serum substitute supplement might improve embryonic development (Hashimoto *et al.*, 2008) and increase implantation and live birth rates, respectively (Meintjes *et al.*, 2009). Despite all these developments, current culture media used in mammalian ART remain suboptimal, given the reduced pregnancy rates, reduced viability and growth and aberrant expression patterns of cultured embryos when compared with *in vivo*-produced embryos (Ho *et*

al., 1995; Walker *et al.*, 1996; Boerjan *et al.*, 2000; Khosla *et al.*, 2001; Bertolini *et al.*, 2002; Rinaudo *et al.*, 2004). Recently, five commercial media systems where compared in a mouse model (Market-Velker *et al.*, 2010). All five culture systems had a varying, but compromised ability to maintain genomic imprinting in comparison with *in vivo*-derived mouse embryos (Market-Velker *et al.*, 2010).

In order to promote embryo growth *in vitro*, culture media are supplemented with growth factors, antioxidants, cytokines and vitamins, while full knowledge of human requirements is lacking. To date, specific concentrations of culture media components are usually proprietary. In the light of the data published here, we urge culture media manufacturers to take their responsibility and disclose the exact composition of IVF culture media systems.

Conclusion and future prospects

There are unfavourable pregnancy and neonatal outcomes after ART for which the underlying factors are still mainly undetermined. Our findings support our hypothesis that culture medium influences perinatal outcome of IVF singletons and twins. This finding has not been confirmed so far for singletons born after FET, although a similar trend was seen. These results indicate that *in vitro* culture might be an important factor explaining the poorer perinatal outcome after ART. LBW is known to be associated with chronic diseases expressed later in life. Because the influence of culture media on human birthweight can be studied and culture media can be changed relatively easily, it is important to investigate their effects. Therefore, validation of our results by data from other IVF clinics is urgently needed. We are currently conducting a multi-centre trial to further investigate the effect of culture media. To optimize embryo culture media, it is crucial not only to determine the morphologic quality of both fresh and frozen embryos, their subsequent implantation and live birth rates and the perinatal outcome, but also to investigate the epigenetic effects in fetal and placental tissues following different ART treatment modalities. This might allow us to select the best embryo culture medium and to minimize or prevent short-term risks and maybe even disease susceptibility in later life.

References

Amaru RC, Bush MC, Berkowitz RL, Lapinski RH,Gaddipati S. Is discordant growth in twins an independent risk factor for adverse neonatal outcome? *Obstet Gynecol* 2004;**103**:(1): 71-76.

Barker DJ. The developmental origins of adult disease. *J Am Coll Nutr* 2004;**23**:(6 Suppl): 588S-595S.

Bertolini M, Beam SW, Shim H, Bertolini LR, Moyer AL, Famula TR,Anderson GB. Growth, development, and gene expression by in vivo- and in vitro-produced day 7 and 16 bovine embryos. *Mol Reprod Dev* 2002;**63**:(3): 318-328.

Biggers JD,Summers MC. Choosing a culture medium: making informed choices. *Fertil Steril* 2008;**90**:(3): 473-483.

Blickstein I,Kalish RB. Birthweight discordance in multiple pregnancy. *Twin Res* 2003;**6**:(6): 526-531.

Boerjan ML, den Daas JH,Dieleman SJ. Embryonic origins of health: long-term effects of IVF in human and livestock. *Theriogenology* 2000;**53**:(2): 537-547.

Bonduelle M, Liebaers I, Deketelaere V, Derde MP, Camus M, Devroey P,Van Steirteghem A. Neonatal data on a cohort of 2889 infants born after ICSI (1991-1999) and of 2995 infants born after IVF (1983-1999). *Hum Reprod* 2002;**17**:(3): 671-694.

Boulet SL, Schieve LA, Nannini A, Ferre C, Devine O, Cohen B, Zhang Z, Wright V,Macaluso M. Perinatal outcomes of twin births conceived using assisted reproduction technology: a population-based study. *Hum Reprod* 2008;**23**:(8): 1941-1948.

De Geyter C, De Geyter M, Steimann S, Zhang H,Holzgreve W. Comparative birth weights of singletons born after assisted reproduction and natural conception in previously infertile women. *Hum Reprod* 2006;**21**:(3): 705-712.

de Mouzon J, Goossens V, Bhattacharya S, Castilla JA, Ferraretti AP, Korsak V, Kupka M, Nygren KG,Nyboe Andersen A. Assisted reproductive technology in Europe, 2006: results generated from European registers by ESHRE. *Hum Reprod* 2010;**25**:(8): 1851-1862.

Dumoulin JC, Derhaag JG, Bras M, Van Montfoort AP, Kester AD, Evers JL, Geraedts JP,Coonen E. Growth rate of human preimplantation embryos is sex dependent after ICSI but not after IVF. *Hum Reprod* 2005;**20**:(2): 484-491.

Dumoulin JC, Land JA, Van Montfoort AP, Nelissen EC, Coonen E, Derhaag JG, Schreurs IL, Dunselman GA, Kester AD, Geraedts JP, *et al.* Effect of in vitro culture of human embryos on birthweight of newborns. *Hum Reprod* 2010;**25**:(3): 605-612.

Fauque P, Mondon F, Letourneur F, Ripoche MA, Journot L, Barbaux S, Dandolo L, Patrat C, Wolf JP, Jouannet P, *et al.* In vitro fertilization and embryo culture strongly impact the placental transcriptome in the mouse model. *PLoS One* 2010;**5**:(2): e9218.

Griesinger G, Kolibianakis EM, Diedrich K,Ludwig M. Ovarian stimulation for IVF has no quantitative association with birthweight: a registry study. *Hum Reprod* 2008;**23**:(11): 2549-2554.

Hambiliki F, Sandell P, Yaldir F,Stavreus-Evers A. A prospective randomized sibling-oocyte study of two media systems for culturing cleavage-stage embryos-impact on fertilization rate. *J Assist Reprod Genet* 2010.

Hansen M, Bower C, Milne E, de Klerk N,Kurinczuk JJ. Assisted reproductive technologies and the risk of birth defects--a systematic review. *Hum Reprod* 2005;**20**:(2): 328-338.

Hansen M, Colvin L, Petterson B, Kurinczuk JJ, de Klerk N,Bower C. Twins born following assisted reproductive technology: perinatal outcome and admission to hospital. *Hum Reprod* 2009;**24**:(9): 2321-2331.

Hashimoto S, Nishihara T, Murata Y, Oku H, Nakaoka Y, Fukuda A,Morimoto Y. Medium without ammonium accumulation supports the developmental competence of human embryos. *J Reprod Dev* 2008;**54**:(5): 370-374.

Hendler I, Goldenberg RL, Mercer BM, Iams JD, Meis PJ, Moawad AH, MacPherson CA, Caritis SN, Miodovnik M, Menard KM, *et al.* The Preterm Prediction Study: association between maternal body mass index and spontaneous and indicated preterm birth. *Am J Obstet Gynecol* 2005;**192**:(3): 882-886.

Hentemann M, Mousavi K,Bertheussen K. Differential pH in embryo culture. *Fertil Steril* 2010.

Ho Y, Wigglesworth K, Eppig JJ,Schultz RM. Preimplantation development of mouse embryos in KSOM: augmentation by amino acids and analysis of gene expression. *Mol Reprod Dev* 1995;**41**:(2): 232-238.

Jackson RA, Gibson KA, Wu YW,Croughan MS. Perinatal outcomes in singletons following in vitro fertilization: a meta-analysis. *Obstet Gynecol* 2004;**103**:(3): 551-563.

Kallen B, Finnstrom O, Lindam A, Nilsson E, Nygren KG,Olausson PO. Selected neonatal outcomes in dizygotic twins after IVF versus non-IVF pregnancies. *Bjog* 2010;**117**:(6): 676-682.

Khosla S, Dean W, Reik W,Feil R. Culture of preimplantation embryos and its long-term effects on gene expression and phenotype. *Hum Reprod Update* 2001;**7**:(4): 419-427.

Koudstaal J, Braat DD, Bruinse HW, Naaktgeboren N, Vermeiden JP,Visser GH. Obstetric outcome of singleton pregnancies after IVF: a matched control study in four Dutch university hospitals. *Hum Reprod* 2000;**15**:(8): 1819-1825.

Land JA. How should we report on perinatal outcome? *Hum Reprod* 2006;**21**:(10): 2638-2639.

Lane M,Gardner DK. Embryo culture medium: which is the best? *Best Pract Res Clin Obstet Gynaecol* 2007;**21**:(1): 83-100.

Mann MR, Lee SS, Doherty AS, Verona RI, Nolen LD, Schultz RM,Bartolomei MS. Selective loss of imprinting in the placenta following preimplantation development in culture. *Development* 2004;**131**:(15): 3727-3735.

Market-Velker BA, Fernandes AD,Mann MR. Side-by-Side Comparison of Five Commercial Media Systems in a Mouse Model: Suboptimal In Vitro Culture Interferes with Imprint Maintenance. *Biol Reprod* 2010;**83**:(6): 938-950.

McDonald SD, Han Z, Mulla S,Beyene J. Overweight and obesity in mothers and risk of preterm birth and low birth weight infants: systematic review and meta-analyses. *Bmj* 2010a;**341**: c3428.

McDonald SD, Han Z, Mulla S, Murphy KE, Beyene J,Ohlsson A. Preterm birth and low birth weight among in vitro fertilization singletons: a systematic review and meta-analyses. *Eur J Obstet Gynecol Reprod Biol* 2009;**146**:(2): 138-148.

McDonald SD, Han Z, Mulla S, Ohlsson A, Beyene J,Murphy KE. Preterm birth and low birth weight among in vitro fertilization twins: a systematic review and meta-analyses. *Eur J Obstet Gynecol Reprod Biol* 2010b;**148**:(2): 105-113.

McIntire DD,Leveno KJ. Neonatal mortality and morbidity rates in late preterm births compared with births at term. *Obstet Gynecol* 2008;**111**:(1): 35-41.

Meintjes M, Chantilis SJ, Ward DC, Douglas JD, Rodriguez AJ, Guerami AR, Bookout DM, Barnett BD,Madden JD. A randomized controlled study of human serum albumin and serum substitute supplement as protein supplements for IVF culture and the effect on live birth rates. *Hum Reprod* 2009;**24**:(4): 782-789.

Oken E, Kleinman KP, Rich-Edwards J,Gillman MW. A nearly continuous measure of birth weight for gestational age using a United States national reference. *BMC Pediatr* 2003;**3**: 6.

Ombelet W, Martens G, De Sutter P, Gerris J, Bosmans E, Ruyssinck G, Defoort P, Molenberghs G,Gyselaers W. Perinatal outcome of 12,021 singleton and 3108 twin births after non-IVF-assisted reproduction: a cohort study. *Hum Reprod* 2006;**21**:(4): 1025-1032.

Palermo G, Joris H, Devroey P,Van Steirteghem AC. Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. *Lancet* 1992;**340**:(8810): 17-18.

Pelinck MJ, Hadders-Algra M, Haadsma ML, Nijhuis WL, Kiewiet SM, Hoek A, Heineman MJ,Middelburg KJ. Is the birthweight of singletons born after IVF reduced by ovarian stimulation or by IVF laboratory procedures? *Reprod Biomed Online* 2010;**21**:(2): 245-251.

Pelkonen S, Koivunen R, Gissler M, Nuojua-Huttunen S, Suikkari AM, Hyden-Granskog C, Martikainen H, Tiitinen A,Hartikainen AL. Perinatal outcome of children born after frozen and fresh embryo transfer: the Finnish cohort study 1995-2006. *Hum Reprod* 2010;**25**:(4): 914-923.

Pinborg A, Loft A, Aaris Henningsen AK, Rasmussen S,Andersen AN. Infant outcome of 957 singletons born after frozen embryo replacement: the Danish National Cohort Study 1995-2006. *Fertil Steril* 2010;**94**:(4): 1320-1327.

Pinborg A, Loft A, Rasmussen S, Schmidt L, Langhoff-Roos J, Greisen G,Andersen AN. Neonatal outcome in a Danish national cohort of 3438 IVF/ICSI and 10,362 non-IVF/ICSI twins born between 1995 and 2000. *Hum Reprod* 2004;**19**:(2): 435-441.

Rimm AA, Katayama AC, Diaz M,Katayama KP. A meta-analysis of controlled studies comparing major malformation rates in IVF and ICSI infants with naturally conceived children. *J Assist Reprod Genet* 2004;**21**:(12): 437-443.

Rinaudo P,Schultz RM. Effects of embryo culture on global pattern of gene expression in preimplantation mouse embryos. *Reproduction* 2004;**128**:(3): 301-311.

Romundstad LB, Romundstad PR, Sunde A, von During V, Skjaerven R, Gunnell D,Vatten LJ. Effects of technology or maternal factors on perinatal outcome after assisted fertilisation: a population-based cohort study. *Lancet* 2008;**372**:(9640): 737-743.

Rooke JA, McEvoy TG, Ashworth CJ, Robinson JJ, Wilmut I, Young LE,Sinclair KD. Ovine fetal development is more sensitive to perturbation by the presence of serum in embryo culture before rather than after compaction. *Theriogenology* 2007;**67**:(3): 639-647.

Rothman KJ, Greenland S,Lash TL (2008). Modern Epidemiology. Philadelphia, Lippincott Williams & Wilkins.

Rydhstroem H. Pregnancy with stillbirth of both twins. *Br J Obstet Gynaecol* 1996;**103**:(1): 25-32.

Sepulveda S, Garcia J, Arriaga E, Diaz J, Noriega-Portella L,Noriega-Hoces L. In vitro development and pregnancy outcomes for human embryos cultured in either a single medium or in a sequential media system. *Fertil Steril* 2009;**91**:(5): 1765-1770.

Steptoe PC,Edwards RG. Birth after the reimplantation of a human embryo. *Lancet* 1978;**2**:(8085): 366.

Suzuki S,Murata T. The influence of assisted reproductive technology on growth discordance in dichorionic twin pregnancies. *Fetal Diagn Ther* 2007;**22**:(5): 372-376.

Swamy GK, Ostbye T,Skjaerven R. Association of preterm birth with long-term survival, reproduction, and next-generation preterm birth. *Jama* 2008;**299**:(12): 1429-1436.

Thomson F, Shanbhag S, Templeton A,Bhattacharya S. Obstetric outcome in women with subfertility. *Bjog* 2005;**112**:(5): 632-637.

Van der Elst J, Camus M, Van den Abbeel E, Maes R, Devroey P,Van Steirteghem AC. Prospective randomized study on the cryopreservation of human embryos with dimethylsulfoxide or 1,2-propanediol protocols. *Fertil Steril* 1995;**63**:(1): 92-100.

Walker SK, Hill JL, Kleemann DO,Nancarrow CD. Development of ovine embryos in synthetic oviductal fluid containing amino acids at oviductal fluid concentrations. *Biol Reprod* 1996;**55**:(3): 703-708.

Wennerholm UB, Soderstrom-Anttila V, Bergh C, Aittomaki K, Hazekamp J, Nygren KG, Selbing A,Loft A. Children born after cryopreservation of embryos or oocytes: a systematic review of outcome data. *Hum Reprod* 2009;**24**:(9): 2158-2172.

Wirleitner B, Vanderzwalmen P, Stecher A, Zech MH, Zintz M,Zech NH. Individual demands of human embryos on IVF culture medium: influence on blastocyst development and pregnancy outcome. *Reprod Biomed Online* 2010;**21**:(6): 776-782.

Young LE, Fernandes K, McEvoy TG, Butterwith SC, Gutierrez CG, Carolan C, Broadbent PJ, Robinson JJ, Wilmut I,Sinclair KD. Epigenetic change in IGF2R is associated with fetal overgrowth after sheep embryo culture. *Nat Genet* 2001;**27**:(2): 153-154.

Zeilmaker GH, Alberda AT, van Gent I, Rijkmans CM,Drogendijk AC. Two pregnancies following transfer of intact frozen-thawed embryos. *Fertil Steril* 1984;**42**:(2): 293-296.

Chapter 3

IVF culture medium affects human intrauterine growth as early as the second trimester of pregnancy

Nelissen ECM, Van Montfoort APA, Smits LJM, Menheere PPCA, Evers JLH, Coonen E, Derhaag JG, Peeters LL, Coumans AB, Dumoulin JCM.

Human Reproduction. 2013;28(8):2067-74.

Presented in part at the 27th annual meeting of the European Society of Human Reproduction and Embryology (ESHRE), Stockholm 2011, Sweden.

Abstract

Study question: When does a difference in human intrauterine growth of singletons conceived after IVF and embryo culture in two different culture media appear?

Summary answer: Differences in fetal development after culture of embryos in one of two IVF media were apparent as early as the second trimester of pregnancy.

What is known already: Abnormal fetal growth patterns are a major risk factor for the development of chronic diseases in adult life. Previously, we have shown that the medium used for culturing embryos during the first few days after fertilization significantly affects the birthweight of the resulting human singletons. The exact onset of this growth difference was unknown.

Study design, size and duration: In this retrospective cohort study, all 294 singleton live births after fresh embryo transfer in the period July 2003 to December 2006 were included. These embryos originated from IVF treatments that were part of a previously described clinical trial. Embryos were allocated to culture in either Vitrolife or Cook commercially available sequential culture media.

Participants/materials, setting, methods: We analysed ultrasound examinations at 8 (n = 290), 12 (n = 83) and 20 weeks' (n = 206) gestation and used first-trimester serum markers [pregnancy-associated plasma protein-A (PAPP-A) and free β-hCG]. Differences between study groups were tested by the Student's t-test, χ^2-test or Fisher's exact test, and linear multivariable regression analysis to adjust for possible confounders (for example, parity, gestational age at the time of ultrasound and fetal gender).

Main results and the role of chance: A total of 294 singleton pregnancies (Vitrolife group $n_{VL} = 168$, Cook group: $n_C = 126$) from 294 couples were included. At 8 weeks' gestation, there was no difference between crow-rump length-based and ovum retrieval-based gestational age (ΔGA) ($n_{VL} = 163$, $n_C = 122$, adjusted mean difference, -0.04 days, $P = 0.84$). A total of 83 women underwent first-trimester screening at 12 weeks' gestation ($n_{VL} = 45$, $n_C = 38$). ΔGA, nuchal translucency (multiples of median, MoM) and PAPP-A (MoM) did not differ between the study groups. Free β-hCG (MoM) ± SEM differed significantly (1.55 ± 0.19 in Vitrolife versus 1.06 ± 0.10 in Cook; $P = 0.031$, Student's *t*-test). At 20

weeks' gestation, a more advanced GA, reflecting an increased fetal growth, was seen at ultrasound examination in the Vitrolife group (n = 115) when compared with the Cook group (n = 91). After adjustment for confounding factors, both the difference between GA based on three biparietal diameter dating formulas minus the actual (ovum retrieval based) GA (adjusted mean difference +1.14 days ($P = 0.04$), +1.14 days ($P = 0.04$) and +1.36 days ($P = 0.048$), as well as head circumference (HC) and trans-cerebellar diameter (TCD) were significantly higher in the Vitrolife group (HC_{vl} 177.3mm, HC_{c} 175.9mm, adjusted mean difference 1.8, $P = 0.03$; TCD_{vl} 20.5mm, TCD_{c} 20.2mm, adjusted mean difference 0.4, $P = 0.008$).

Limitations, reasons for caution: A first-trimester (12 weeks) fetal screening was not yet offered routinely during the study period, therefore only 28% of women in our study participated in this elective screening programme. Although all sonographers were experienced and specially trained to perform these ultrasound examinations and were unaware of the randomization procedure, we cannot totally rule out possible intra- and inter-observer variability. Despite being indispensable in daily practice, sonographic weight formulas have a limited accuracy.

Wider implications of the findings: According to the fetal origins hypothesis, many adult diseases originate *in utero* owing to adaptations made by the fetus to the environment it encounters. This study indicates that the embryonic environment is already important for fetal development. Therefore, our study emphasizes the need to investigate fetal growth patterns after assisted reproduction technologies and long-term health outcomes of IVF children, especially in relation to the culture medium used during the first few days of preimplantation development.

Introduction

In our previous studies, we have shown that the medium used for culturing IVF embryos during the first few days of preimplantation development affects birthweight of the resulting human singletons (Dumoulin *et al.* 2010; Nelissen *et al.* 2012). Even after correction for a large range of possible confounders, such as gestational age (GA) at delivery, fetal gender, number of transferred embryos, day of embryo transfer (second or third), fertilization method (IVF or ICSI), parity, parental height, weight and age, cause and duration of infertility, pregnancy related factors and life-style factors (e.g. smoking), the significant association between human birthweight and type of culture medium persisted (Dumoulin *et al.* 2010; Nelissen *et al.* 2012). The time of onset of this growth difference is, as yet, unknown.

Increasing evidence indicates that pace and pathway of fetal growth is a major risk factor for the development of chronic diseases in adult life, such as hypertension, type 2 diabetes, coronary heart disease and stroke, often referred to as the "fetal origins hypothesis" (Barker *et al.* 2009). Although the general IVF progeny is still too young to determine the prevalence of these adult diseases, in IVF children parameters of metabolic syndrome, like increased systolic and diastolic blood pressures and glucose levels, are reported (Ceelen *et al.* 2008).

Evidence is accumulating that factors in the peri-conceptional period, such as maternal undernutrition, can modify the fetal growth trajectories, sometimes even with negligible effect on birthweight but nevertheless with adverse consequences for long-term health (Bloomfield *et al.* 2006). This suggests that the presence of appropriate nutrients, and other factors, in IVF culture media will be vital for embryonic development during this high-risk period. In the light of increasing numbers of children born worldwide after a pregnancy established by assisted reproduction technologies (ARTs), exploration of this topic is of pivotal importance.

The aim of the present study was to increase our insight into the impact of embryo culture media on subsequent fetal growth patterns, with particular focus on the onset of growth divergence. We hypothesized that the effect of *in vitro* culture of embryos on fetal growth will increase during the first trimester and will be detectable at the end of the first trimester or during the second trimester, because these differences will be the result of modified growth trajectories of the fetus itself after *in vitro* culture in different media, rather than differences in parental or other external factors. To examine this, we

evaluated ultrasound examinations performed at 8, 12 and 20 weeks' gestation in patients who participated in our previous study (Nelissen *et al.* 2012) and used first-trimester serum markers [free β-hCG (fβ-hCG) and pregnancy-associated plasma protein-A (PAPP-A)], as indicators of placental function.

Materials and methods

Study population

At the Maastricht University Medical Centre (MUMC), we studied 294 singleton live births after fresh embryo transfer, from a previously reported comparative study (Nelissen *et al.* 2012). In this cohort, IVF (or IVF with ICSI) was applied in the period between July 2003 and December 2006 during which two widely used commercially available sequential culture media [Vitrolife G1.3 (Göteborg, Sweden) or Cook K-SICM (Brisbane, Australia)] were used in alternating order. For this study, all singletons born alive after the 20th week of gestation, who were a couple's first child from an IVF treatment performed during the study period, were included in the analysis. Data on pregnancy outcome including complications, such as gestational diabetes, hypertension and preeclampsia, and perinatal outcome, were collected from their obstetricians or midwives. Exclusion criteria for the study were patients who applied for PGD or required donor oocytes. All participating couples gave written informed consent for the use of their anonymized data.

Culture medium allocation and IVF procedure

We strictly alternated treatment allocation to either of the two media on the day before the ovum retrieval. This was performed by laboratory technicians who were unaware of patient characteristics. The order of ovum retrievals on a certain day, which determined the allocation to one of the study groups, was planned by clinical personnel who were unaware of the laboratory allocation procedure. This allocation to one of the two media was part of our internal quality monitoring system in order to be able to identify suboptimal batches of a particular medium. Except for the media, all other IVF procedures (clinical as well as laboratory) were similar in both groups. Detailed ovarian stimulation, fertilization, culture and fresh embryo transfer procedures have been described previously (Dumoulin *et al.* 2010).

Fetal growth data collection

GA was calculated from the day of oocyte retrieval, which was defined as Day 14 of the cycle. In the Netherlands, all pregnant women are offered ultrasound dating during the first trimester and are counselled for fetal ultrasound examination at 20 weeks' gestation to have fetal biometry performed and be screened for structural abnormalities. For the present study, the results of ultrasonic measurements were collected retrospectively and all sonographers were unaware of the randomization procedure and its outcome. Furthermore, all sonographers were experienced and specially trained for these ultrasound examinations.

In viable pregnancies, fetal crown-rump length (CRL) was measured at 7-8 weeks' gestation in a mid-sagittal section of the embryo using transvaginal ultrasonography with care being taken to avoid inclusion of the yolk sac. The difference between the CRL-based GA calculated with the formula of Hadlock (1992) minus the actual (ovum retrieval based) GA, was expressed as the difference in days of gestation (ΔGA) (Hadlock *et al.* 1992).

Only a subset of women participated in (elective) first-trimester screening for Down syndrome (information about the possibility for screening has only been given to all pregnant women in the Netherlands since June 2004). In these cases, serum was sampled at a gestational interval ranging from 11 to 13 weeks plus 6 days (NICE *et al.* 2008). The concentrations of maternal serum fβ-hCG and PAPP-A were analyzed with commercially available kits and the AutoDELFIA analyser (PerkinElmer, Turku, Finland) at the National Institute for Public Health and the Environment (Bilthoven, The Netherlands) during the years 2003-2005 and at the authorized clinical chemistry laboratory of the MUMC during the years 2006-2007. CRL and fetal nuchal translucency (NT) thickness were obtained during abdominal ultrasonography. The values of fβ-hCG, PAPP-A and NT were expressed as multiples of the median (MoM) for GA with corrections for maternal weight according to the guidelines of the national first trimester prenatal screening programme (Schielen *et al.* 2003).

Furthermore, several sonographic parameters were measured during the mid-trimester ultrasound scan at ~20 weeks' gestation to estimate GA and to calculate estimated fetal weight (EFW). The fetal biparietal diameter (BPD), head circumference (HC), abdominal circumference (AC), femur length (FL) and trans-cerebellar diameter (TCD) were measured using standardized ultrasound procedures. BPD dating formulas of Mul *et al.* (#1 and #2) (1996) and Selbing

and Kjessler (1985) were used to calculate the GA (Saltvedt *et al.* 2004). The difference between GA calculated by BPD dating formulas minus the actual (ovum retrieval based) GA was expressed in days (ΔGA). EFW was calculated using the formulas of Hadlock I (BPD, HC, AC, FL), Hadlock III (BPD, AC, FL) and Hadlock IV (HC, AC, FL) (Hadlock *et al.* 1985; Hoopmann *et al.* 2010).

Statistical analysis

Crude differences between study groups were tested using the Student's *t*-test for continuous variables and the χ^2-test for binary variables (Fisher's exact test in case of less than five observations per cell). *P*-values of <0.05 (two-sided testing) were considered to reflect statistical significance. Linear regression analysis was applied in order to control for any between-group differences with respect to other determinants of fetal growth. In one of our previous articles (Dumoulin *et al.,* 2010), we found a difference between study groups in height and weight, but not BMI. Therefore, we used height and weight instead of BMI in our multiple regression analyses. The following factors were included in all multivariable analyses: parity, GA at the time of ultrasound, characteristics of both parents (age, weight, height, daily number of cigarettes smoked), duration of infertility, cause of infertility, pregnancy complications (gestational diabetes, hypertension and pre-eclampsia), day of embryo transfer (second or third), number of transferred embryos (one or two) and fetal gender. The residuals were normally distributed in all regression analyses. Residual versus fitted plots did not indicate heteroscedasticity. Furthermore, there was no evidence of collinearity.

Results

A total of 294 singleton pregnancies (168 in the Vitrolife group versus 126 in the Cook group) from 294 different couples were included. Neonatal characteristics have been reported previously (Nelissen *et al.* 2012). Parental characteristics are listed in Table I. The distribution of the confounding variables is described in Supplementary data, Table I (available online). The rate of pregnancy complications (such as gestational diabetes, hypertension and pre-eclampsia), was similar in both groups (14 (8.3%) in the Vitrolife group versus 9 (7.1%) in the Cook group).

Table I Parental characteristics of singletons born after fresh embryo transfers.

Characteristic	8 weeks		12 weeks		20 weeks	
	Vitrolife group (n=167)	Cook group (n= 123)	Vitrolife Group (n=45)	Cook Group (n=38)	Vitrolife Group (n=115)	Cook Group (n=91)
Maternal characteristics						
Age (years)	32.4 ± 4.0	32.6 ± 3.6	34.0 ± 4.1	33.6 ± 3.4	32.4 ± 4.0	32.6 ± 3.6
Age > 38 years	11 (6.6)	6 (4.9)	7 (15.6)	4 (10.5)	8 (7.0)	6 (6.6)
Height (cm)	169.4 ± 5.8	167.4 ± 7.3	168.6 ± 6.2	168.7 ± 8.4	169.6 ± 5.8	167.5 ± 7.5
Weight (kg)	70.1 ± 10.5	66.7 ± 9.8	69.0 ± 9.8	68.0 ± 12.7	70.8 ± 10.9	66.7 ± 9.8
Body mass index (range)	24.4 (17.5 – 33.3)	23.8 (18.0 – 31.4)	24.3 (3.0)	23.8 (3.4)	24.6 (3.5)	23.8 (3.0)
Smoking ≥ 10 cigarettes/day	25 (15.0)	17 (13.8)	6 (13.3)	4 (10.5)	16 (13.9)	11 (12.1)
Paternal characteristics						
Age (years)	35.3 ± 5.7	35.7 ± 4.9	35.9 ± 6.9	37.7 ± 5.5	35.1 ± 4.9	36.1 ± 5.2
Height (cm)	182.1 ± 7.8	180.8 ± 7.4	182.3 ± 7.6	182.2 ± 7.6	181.9 ± 7.5	181.6 ± 7.1
Weight (kg)	86.4 ± 13.9	83.0 ± 11.2	88.3 ± 14.7	85.2 ± 11.1	85.9 ± 13.8	83.7 ± 10.8
Body mass index (range)	26.0 (17.9 – 46.0)	25.3 (19.9 – 34.3)	26.6 (4.0)	25.6 (2.8)	25.9 (3.6)	25.3 (2.7)
Smoking ≥ 10 cigarettes/day	29 (17.4)	25 (20.3)	5 (11.1)	7(18.4)	20 (17.4)	15 (16.5)
Primary indication for IVF treatment						
Tubal factor	17 (10.2)	15 (12.2)	8 (17.8)	4 (10.5)	8 (7.0)	10 (11.0)
Male factor	107 (64.1)	79 (64.2)	29 (64.4)	27 (71.1)	86 (74.8)	65 (71.4)
Unexplained	36 (21.6)	25 (20.3)	8 (17.8)	5 (13.2)	15 (13.0)	12 (13.2)
Other	7 (4.2)	4 (3.3)	0	2 (5.3)	6 (5.2)	4 (4.4)

Data are presented as numbers (%) or mean ±SD.

8 weeks' gestation

Fetal CRL was measured around 8 weeks' gestation (range 6 weeks and 5 days to 9 weeks and 2 days) in 290 singleton pregnancies, 167 in the Vitrolife group and 123 in the Cook group. Four couples were lost to follow-up. The ΔGA was comparable in the two groups (unadjusted mean difference -0.08 days, $P = 0.68$, adjusted -0.04 days, $P = 0.84$) (Table II).

Table II Difference in gestational age at 8 and 12 weeks' gestation.

	Vitrolife group (n=168)	**Cook group (n=126)**	**Unadjusted mean difference**	***P*-value**	**Adjusted mean difference**	***P*-value**
8 weeks' gestation	-0.42 (n=167)	-0.50 (n= 123)	-0.08	0.68	-0.04	0.84
12 weeks' gestation	1.80 (n=45)	1.38 (n=38)	-0.42	0.58	-0.59	0.22

Data are presented as days. Difference between crown-rump-length based gestational age (GA) minus actual (ovum retrieval based) GA, was expressed as difference in days
of gestation (ΔGA). A negative value indicates an underestimation of the GA, while a positive value indicates an overestimation of the GA. Unadjusted mean differences are
calculated using the Student's *t*-test and adjusted mean differences using linear multivariable regression analysis.

12 weeks' gestation

A total of 83 women chose to undergo first-trimester screening around 12 weeks' gestation (range 11 weeks and 3 days to 13 weeks and 6 days): 45 in the Vitrolife group (27%) and 38 in the Cook group (30%). The difference in ΔGA (adjusted mean difference -0.59 days, $P = 0.22$) (Table II) and that in NT and PAPP-A was statistically insignificant (Table III). However, fβ-hCG and fβ-hCG (MoM) in the Vitrolife group were significantly higher than in the Cook group ($P = 0.029$, $P = 0.031$, respectively. Table III).

Table III Ultrasound examination and serum markers at 12 weeks' gestation.

	Vitrolife group (n=45)	**Cook group (n=38)**	***P*-value**
NT(MoM)	0.78 ± 0.10	0.65 ± 0.03	0.237
PAPP-A (MoM)	1.03 ± 0.09	1.05 ± 0.09	0.874
fβ-hCG	53.5 ± 6.03	37.2 ± 3.61	0.029
fβ-hCG (MoM)	1.55 ± 0.19	1.06 ±0.10	0.031

Data are presented as mean ± SEM. NT, fetal nuchal translucency thickness; MoM, multiples of median; fβ-hCG, maternal serum free β-human chorionic gonadotrophin; PAPP-A, pregnancy-associated plasma protein-A.

20 weeks' gestation

Fetal growth parameters were measured around 20 weeks' gestation (range 18 weeks and 2 days to 22 weeks and 1 day) in 206 pregnancies, 115 (68%) in the Vitrolife group and 91 (72%) in the Cook group. The remaining 88 couples chose not to have the mid-trimester fetal ultrasound scan or had this examination elsewhere in the Netherlands. Using 3 distinct BPD dating formulas, the adjusted ΔGA differed significantly between groups in all 3 calculations. In the Vitrolife group, all ΔGA were more positive than in the Cook group (linear multivariable regression analysis; $P = 0.04$, $P = 0.04$ and $P = 0.048$) (Table IV). This is consistent with a more advanced GA, reflecting an increased fetal growth in the Vitrolife group. The adjusted mean values of several other sonographic parameters (BPD, HC, AC, FL and TCD) are shown in Table V. All these fetal sonographic parameters are useful to assess fetal growth. HC and TCD were significantly higher in the Vitrolife group compared with the Cook group ($P = 0.03$, $P = 0.008$, respectively), again reflecting an increased fetal growth in the Vitrolife group. EFW, as calculated with Hadlock formulas I, III or IV, did not differ between the two groups at 20 weeks' gestation (Table V).

Table IV Difference in gestational age at 20 weeks' gestation using BPD dating formulas.

	Vitrolife group (n=115)	Cook group (n=91)	Unadjusted mean difference	*P*-value	Adjusted mean difference	*P*-value
Mull *et al.* 1996 #1	+3.28	+2.10	1.18	0.04	1.14	0.04
Mull *et al.* 1996 #2	+2.28	+1. 10	1.18	0.04	1.14	0.04
Selbing & Kjessler, 1985	+6.18	+4.77	1.41	0.05	1.36	0.048

Data are presented as days. Difference between GA calculated with biparietal diameter (BPD) dating formulas minus the actual (ovum retrieval based) GA, was expressed in days (ΔGA). Unadjusted mean differences are calculated using the Student's *t*-test and adjusted mean differences using linear multivariable regression analysis.

Table V Difference in sonographic parameters and estimated fetal weight (EFW) at 20 weeks' gestation.

	Vitrolife group (n=115)	Cook group (n=91)	Unadjusted mean difference	*P*-value	Adjusted mean difference	*P*-value
BPD	50.2 (0.3)	49.8 (0.3)	0.4	0.27	0.5	0.07
AC	152.1 (0.9)	151.2 (1.0)	0.9	0.51	0.8	0.42
HC	177.3 (0.8)	175.9 (0.9)	1.4	0.24	1.8	0.03
FL	32.7 (0.2)	32.8 (0.3)	-0.1	0.80	-0.1	0.83
TCD	20.5 (0.1)	20.2 (0.1)	0.3	0.11	0.4	0.008
EFW (Hadlock I) BPD, HC, AC, FL	350.1 (4.1)	347.9 (4.9)	2.27	0.72	2.62	0.53
EFW (Hadlock III) BPD, AC, FL	357.3 (4.3)	354.8 (5.0)	2.53	0.70	2.83	0.51
EFW (Hadlock IV) HC, AC, FL	346.0 (4.1)	343.6 (4.8)	2.44	0.70	2.82	0.49

Data are presented as mean values (mm or gram) (±SEM). BPD, biparietal diameter; HC, head circumference; AC, abdominal circumference; FL, femur length; TCD, transcerebellar diameter. Unadjusted mean differences are calculated using the Student's *t*-test and adjusted mean differences using linear multivariable regression analysis.

Discussion

The main findings of our study of a series of singletons are that the Vitrolife group differed from the Cook group by significantly higher circulating levels of fβ-hCG (MoM) at 12 weeks and a more advanced GA, consistent with an increased fetal growth, at 20 weeks' gestation. In previous studies, we observed a significant difference in birthweight between the two study groups of singletons resulting from culture in either Vitrolife or Cook medium (Dumoulin *et al.* 2010; Nelissen *et al.* 2012). Although in these studies, we controlled for many maternal and environmental co-variables specified in our analysis, theoretically it could be possible that our results were influenced by the effect of an as yet unknown maternal or pregnancy related confounder. It is generally assumed that during the first half of pregnancy, the fetus' own genetic programme is the primary determinant of growth, while during the second half of pregnancy, many external factors, such as maternal factors (pre-pregnancy weight or BMI, height, ethnic background), pregnancy-related factors (gestational diabetes, hypertension and pre-eclampsia) and environmental factors (maternal nutrition, disease, smoking, drugs) have an increasing impact on fetal growth (Mongelli *et al.* 1995; Rosenberg *et al.* 2005; Bergsjo *et al.* 2007; Boron *et al.* 2009; Gaillard *et al.* 2011). As the results of the present study indicate that the growth differences between

the study groups are already discernible in the second trimester of pregnancy, our findings of the differences in birthweight between the two study groups are more in line with our hypothesis that these differences are the result of modified growth trajectories of the fetus itself after *in vitro* culture in different media, rather than differences in parental or other external factors.

Recently, variability in first-trimester fetal growth by maternal factors was investigated in humans (Mook-Kanamori *et al.* 2010). Fetal CRL appeared positively associated with higher maternal age, parity >1, folic acid use, negatively associated with, for example, smoking and not associated with maternal anthropometrics (Mook-Kanamori *et al.* 2010). After adjustment for these and several other confounding factors (except folic acid because of standard use with IVF treatment), we found no significant ΔGA between groups at 8 weeks' gestation.

Measurement of fetal CRL using high-frequency transvaginal ultrasound for early pregnancy dating is accurate (Verburg *et al.* 2008; Papaioannou *et al.* 2010). In our study, at 12 weeks' gestation, no significant difference in ΔGA, PAPP-A and NT was found between the two study groups. Bukowski *et al.* (2007) found that ART pregnancies with appropriate fetal growth (ΔGA=0) in the first trimester had lower birthweight and a higher risk of small-for-gestational age infants than the general population (Bukowski *et al.* 2007). Also Conway *et al.* (2011) did not find an appreciable difference in first-trimester fetal growth as measured by CRL between spontaneously conceived and ART pregnancies. The findings from both studies suggest that the generally reported impaired fetal growth in ART pregnancies (Helmerhorst *et al.* 2004; Jackson *et al.* 2004; McDonald *et al.* 2009), becomes only detectable after the first trimester, which is in line with our results.

At 20 weeks' gestation, an increased fetal growth is seen in the Vitrolife group when compared with the Cook group, which corresponds to the also significantly higher birthweight in this group detailed in our previous report (Nelissen *et al.* 2012). After adjustment for confounding factors, ΔGA (based on BPD dating formulas), HC and TCD were significantly higher in the Vitrolife group. The TCD increases by ~1 mm per week between a GA of 14 and 21 weeks (ISUOG 2007). The adjusted mean difference of 0.4 mm indicates that fetal size in the Vitrolife group was consistent with a GA of ~3 days longer than the Cook group. The clinical relevance of these differences and the forthcoming difference in birthweight is unknown. However, our results indicate that maintaining embryos

in culture medium for only 2-3 days can result in a difference in fetal growth rate. To calculate the GA, we used the BPD dating formulas of Mul *et al.* (#1 and #2) (1996) and Selbing and Kjessler (1985), as these were based on measurements in IVF pregnancies and they performed well with small systematic and random errors at late dating (15-20 gestational weeks) in an IVF population (Saltvedt *et al.* 2004). The variance in AC depends mainly on the abdominal fat tissue and furthermore on the volume of the liver, stomach or spleen (Kehl *et al.* 1996). Fat deposition begins from ~24 gestational weeks (Cunningham *et al.* 2010). At 20 weeks' gestation, the amount of abdominal fat tissue is still low which could explain why we did not find a difference in AC at this stage in fetal development.

There is no preferred formula to estimate fetal weight and sonographic weight formulas generally show poor rates of accuracy (Dudley 2005). Nonetheless, fetal weight estimation is important for obstetric care management. Most formulas are derived from non-linear regression analysis using single or combined ultrasound measurements, whereas others are based on volumetric methods. Mostly used are the formulas of Hadlock (Hadlock *et al.* 1985; Hoopmann *et al.* 2010). We used the formulas of Hadlock I, III and IV, and showed no significant difference in EFW between groups. This was explainable as AC and FL, which were similar between groups, have a higher contribution in the EFW regression model of Hadlock than HC and/or BPD.

Our study has limitations with respect to the allocation procedure, being a strictly alternate-case, quasi-random one. However, as allocation was performed with two-sided allocation concealment and blinding, this alternate allocation to one of two media closely approaches optimal randomisation. Furthermore, all sonographers were unaware of the randomization procedure, because ultrasound measurements were collected retrospectively for this study. Although all sonographers were experienced and specially trained to perform these ultrasound examinations, we cannot totally rule out a possible intra- and inter-observer variability. Owing to the fact that a first-trimester (12 weeks) fetal screening was not yet offered routinely during the study period, only 28% of women in our study participated in this elective screening programme.

More and more studies are now investigating adverse influences in the preimplantation embryonic stage upon fetal growth and development. It appears that peri-conceptional undernutrition in sheep can alter fetal growth trajectories (Rumball *et al.* 2009). In rats, a maternal low protein diet only during the pre-implantation period resulted in lower cell numbers in the inner cell mass

and trophectoderm, reduced birthweight and hypertension in the offspring (Kwong *et al.* 2000) and altered expression of growth-regulating imprinted genes (Kwong *et al.* 2006). A maternal low protein diet fed exclusively during mouse preimplantation development had no appreciable effect on fetal growth but was associated with excess post-natal growth and sustained hypertension (Watkins *et al.* 2008).

Also effects on fetal development related to *in vitro* culture were found in several animal models (Farin *et al.* 2006; Delle Piane *et al.* 2010). However, not only fetal development but also placental development is affected. In several animal models, ART has been shown to lead to a diverse set of placental abnormalities, such as an impaired placental steroid metabolism in mice (Collier *et al.* 2009), alterations in placentome morphology and blood vessels in cattle (Farin *et al.* 2006) and a larger placental/fetal weight ratio in mice (Delle Piane *et al.* 2010). In human, an increased risk of placental abnormalities, such as placenta praevia, has been shown (Kallen *et al.* 2005; Romundstad *et al.* 2006). One can postulate that these placental abnormalities may partly explain the obstetric and neonatal complications associated with ART.

In our present series of IVF singletons, fβ-hCG (MoM) measured at around 12 weeks'gestation was found to be significantly higher in the Vitrolife than the Cook group. hCG is a glycoprotein produced by the syncytiotrophoblast cells (Kovalevskaya *et al.* 2002). Levels of hCG increase rapidly in early pregnancy until peak levels are reached at 7 to 9 weeks, followed by a progressive decline until around 20 weeks and then remain comparatively low and stable until term (Kletzky *et al.* 1985). hCG plays a major role in early human development, especially in the first trimester but also during the remainder of pregnancy. For instance hCG promotes progesterone production, angiogenesis in uterine vasculature, growth of the uterus, differentiation of growing cytotrophoblast cells, quiescence of myometrial contractions and has a function in growth and development of fetal organs (Keay *et al.* 2004; Ticconi *et al.* 2007; Cole 2010). Furthermore, low first-trimester fβ-hCG levels are associated with fetal growth restriction, gestational hypertension and gestational diabetes (Ong *et al.* 2000), and with SGA (Dugoff *et al.* 2004; Krantz *et al.* 2004; Kirkegaard *et al.* 2011; Poon *et al.* 2011). The effect of ART on first trimester fβ-hCG levels has been extensively studied indicating increased, decreased or unaltered levels (for review see Gjerris *et al.* 2012). Our increased fβ-hCG (MoM) level in the Vitrolife group is consistent with an increased fetal growth seen in the Vitrolife group. Orasanu

et al. (2006) investigated the effect of four different IVF culture media on serum hCG concentrations measured on Day 15 after embryo transfer in singleton viable pregnancies. Vitrolife G1.3 medium was associated with higher serum hCG levels on Day 15 after embryo transfer compared with the other media. However, the average of 3.5 embryos transferred could have led to undetected early implantation sites (Orasanu *et al.* 2006).

Since the level of fβ-hCG in early pregnancy is assumed to represent the mass of syncytial trophoblast (Almog *et al.* 2011), it is possible that the number of trophoblast cells is increased after culture in Vitrolife medium compared with Cook medium. Effects of IVF on the number of trophoblast cells in humans have been suggested previously (Turan *et al.* 2010). Also, in mice and rat models, embryo culture or a maternal low-protein diet around conception leads to altered cell numbers in the trophectoderm and inner cell mass (Kwong *et al.* 2000; Watkins *et al.* 2007). Changes in epigenetic regulation might explain the altered cell numbers as well as differences in hCG level and/or fetal growth as this plays an important role in placental development and function, and can be disturbed by ART (Nelissen *et al.* 2011; van Montfoort *et al.* 2012). From animal studies it is known that culture medium can affect the epigenetic regulation of imprinted genes, mainly in placental tissue, and to a lesser extent in the embryo (Mann *et al.* 2004; Rivera *et al.* 2008; Fauque *et al.* 2010). A possible explanation for our finding might be that Vitrolife G1.3 medium modulates the epigenetic regulation of the placenta leading to increased fβ-hCG levels. However, ours is a relatively small study and our observations need to be investigated in larger studies before firm clinical conclusions can be drawn.

Conclusion and future perspective

According to the fetal origins hypothesis, many adult diseases originate *in utero* owing to adaptations made by the fetus to the environment it encounters. Our study shows that IVF culture medium has the ability to influence fetal growth rate. This emphasizes the need for studies investigating fetal growth patterns after ART and the long-term health outcomes of these IVF children.

References

Almog B, Al-Shalaty J, Sheizaf B, Shehata F, Son WY, Tan SL,Tulandi T. Difference between serum beta-human chorionic gonadotropin levels in pregnancies after in vitro maturation and in vitro fertilization treatments. *Fertil Steril* 2011;**95**:(1): 85-88.

Barker DJ, Osmond C, Kajantie E,Eriksson JG. Growth and chronic disease: findings in the Helsinki Birth Cohort. *Ann Hum Biol* 2009;**36**:(5): 445-458.

Bergsjo P, Bakketeig LS,Lindmark G. Maternal smoking does not affect fetal size as measured in the mid-second trimester. *Acta Obstet Gynecol Scand* 2007;**86**:(2): 156-160.

Bloomfield FH, Oliver MH,Harding JE. The late effects of fetal growth patterns. *Arch Dis Child Fetal Neonatal Ed* 2006;**91**:(4): F299-304.

Boron WF,Boulpaep EL, Eds. (2009). Medical Physiology: a cellular and molecular approach. Chapter 57, fetal and neonatal physiology. Philadelphia, Saunders Elsevier.

Bukowski R, Smith GC, Malone FD, Ball RH, Nyberg DA, Comstock CH, Hankins GD, Berkowitz RL, Gross SJ, Dugoff L*, et al.* Fetal growth in early pregnancy and risk of delivering low birth weight infant: prospective cohort study. *Bmj* 2007;**334**:(7598): 836.

Ceelen M, van Weissenbruch MM, Vermeiden JP, van Leeuwen FE,Delemarre-van de Waal HA. Cardiometabolic differences in children born after in vitro fertilization: follow-up study. *J Clin Endocrinol Metab* 2008;**93**:(5): 1682-1688.

Cole LA. Hyperglycosylated hCG, a review. *Placenta* 2010;**31**:(8): 653-664.

Collier AC, Miyagi SJ, Yamauchi Y,Ward MA. Assisted reproduction technologies impair placental steroid metabolism. *J Steroid Biochem Mol Biol* 2009;**116**:(1-2): 21-28.

Conway DA, Liem J, Patel S, Fan KJ, Williams J, 3rd,Pisarska MD. The effect of infertility and assisted reproduction on first-trimester placental and fetal development. *Fertil Steril* 2011;**95**:(5): 1801-1804.

Cunningham FG, Leveno KJ, Bloom SL, Hauth JC, Rouse DJ,Spong CY (2010). Williams Obstetrics, The McGraw-Hill Companies.

Delle Piane L, Lin W, Liu X, Donjacour A, Minasi P, Revelli A, Maltepe E,Rinaudo PF. Effect of the method of conception and embryo transfer procedure on mid-gestation placenta and fetal development in an IVF mouse model. *Hum Reprod* 2010;**25**:(8): 2039-2046.

Dudley NJ. A systematic review of the ultrasound estimation of fetal weight. *Ultrasound Obstet Gynecol* 2005;**25**:(1): 80-89.

Dugoff L, Hobbins JC, Malone FD, Porter TF, Luthy D, Comstock CH, Hankins G, Berkowitz RL, Merkatz I, Craigo SD*, et al.* First-trimester maternal serum PAPP-A and free-beta subunit human chorionic gonadotropin concentrations and nuchal translucency are associated with obstetric complications: a population-based screening study (the FASTER Trial). *Am J Obstet Gynecol* 2004;**191**:(4): 1446-1451.

Dumoulin JC, Land JA, Van Montfoort AP, Nelissen EC, Coonen E, Derhaag JG, Schreurs IL, Dunselman GA, Kester AD, Geraedts JP*, et al.* Effect of in vitro culture of human embryos on birthweight of newborns. *Hum Reprod* 2010;**25**:(3): 605-612.

Farin PW, Piedrahita JA,Farin CE. Errors in development of fetuses and placentas from in vitro-produced bovine embryos. *Theriogenology* 2006;**65**:(1): 178-191.

Fauque P, Mondon F, Letourneur F, Ripoche MA, Journot L, Barbaux S, Dandolo L, Patrat C, Wolf JP, Jouannet P*, et al.* In vitro fertilization and embryo culture strongly impact the placental transcriptome in the mouse model. *PLoS One* 2010;**5**:(2): e9218.

Gaillard R, de Ridder MA, Verburg BO, Witteman JC, Mackenbach JP, Moll HA, Hofman A, Steegers EA,Jaddoe VW. Individually customised fetal weight charts derived from ultrasound measurements: the Generation R Study. *Eur J Epidemiol* 2011;**26**:(12): 919-926.

Gjerris AC, Tabor A, Loft A, Christiansen M,Pinborg A. First trimester prenatal screening among women pregnant after IVF/ICSI. *Hum Reprod Update* 2012.

Hadlock FP, Harrist RB, Sharman RS, Deter RL,Park SK. Estimation of fetal weight with the use of head, body, and femur measurements--a prospective study. *Am J Obstet Gynecol* 1985;**151**:(3): 333-337.

Hadlock FP, Shah YP, Kanon DJ,Lindsey JV. Fetal crown-rump length: reevaluation of relation to menstrual age (5-18 weeks) with high-resolution real-time US. *Radiology* 1992;**182**:(2): 501-505.

Helmerhorst FM, Perquin DA, Donker D,Keirse MJ. Perinatal outcome of singletons and twins after assisted conception: a systematic review of controlled studies. *Bmj* 2004;**328**:(7434): 261.

Hoopmann M, Abele H, Wagner N, Wallwiener D,Kagan KO. Performance of 36 different weight estimation formulae in fetuses with macrosomia. *Fetal Diagn Ther* 2010;**27**:(4): 204-213.

ISUOG. Sonographic examination of the fetal central nervous system: guidelines for performing the 'basic examination' and the 'fetal neurosonogram'. *Ultrasound Obstet Gynecol* 2007;**29**:(1): 109-116.

Jackson RA, Gibson KA, Wu YW,Croughan MS. Perinatal outcomes in singletons following in vitro fertilization: a meta-analysis. *Obstet Gynecol* 2004;**103**:(3): 551-563.

Kallen B, Finnstrom O, Nygren KG, Otterblad Olausson P,Wennerholm UB. In vitro fertilisation in Sweden: obstetric characteristics, maternal morbidity and mortality. *Bjog* 2005;**112**:(11): 1529-1535.

Keay SD, Vatish M, Karteris E, Hillhouse EW,Randeva HS. The role of hCG in reproductive medicine. *Bjog* 2004;**111**:(11): 1218-1228.

Kehl RJ, Krew MA, Thomas A,Catalano PM. Fetal growth and body composition in infants of women with diabetes mellitus during pregnancy. *J Matern Fetal Med* 1996;**5**:(5): 273-280.

Kirkegaard I, Henriksen TB,Uldbjerg N. Early fetal growth, PAPP-A and free beta-hCG in relation to risk of delivering a small-for-gestational age infant. *Ultrasound Obstet Gynecol* 2011;**37**:(3): 341-347.

Kletzky OA, Rossman F, Bertolli SI, Platt LD,Mishell DR, Jr. Dynamics of human chorionic gonadotropin, prolactin, and growth hormone in serum and amniotic fluid throughout normal human pregnancy. *Am J Obstet Gynecol* 1985;**151**:(7): 878-884.

Kovalevskaya G, Genbacev O, Fisher SJ, Caceres E,O'Connor JF. Trophoblast origin of hCG isoforms: cytotrophoblasts are the primary source of choriocarcinoma-like hCG. *Mol Cell Endocrinol* 2002;**194**:(1-2): 147-155.

Krantz D, Goetzl L, Simpson JL, Thom E, Zachary J, Hallahan TW, Silver R, Pergament E, Platt LD, Filkins K, *et al.* Association of extreme first-trimester free human chorionic gonadotropin-beta, pregnancy-associated plasma protein A, and nuchal translucency with intrauterine growth restriction and other adverse pregnancy outcomes. *Am J Obstet Gynecol* 2004;**191**:(4): 1452-1458.

Kwong WY, Miller DJ, Ursell E, Wild AE, Wilkins AP, Osmond C, Anthony FW,Fleming TP. Imprinted gene expression in the rat embryo-fetal axis is altered in response to periconceptional maternal low protein diet. *Reproduction* 2006;**132**:(2): 265-277.

Kwong WY, Wild AE, Roberts P, Willis AC,Fleming TP. Maternal undernutrition during the preimplantation period of rat development causes blastocyst abnormalities and programming of postnatal hypertension. *Development* 2000;**127**:(19): 4195-4202.

Mann MR, Lee SS, Doherty AS, Verona RI, Nolen LD, Schultz RM,Bartolomei MS. Selective loss of imprinting in the placenta following preimplantation development in culture. *Development* 2004;**131**:(15): 3727-3735.

McDonald SD, Han Z, Mulla S, Murphy KE, Beyene J,Ohlsson A. Preterm birth and low birth weight among in vitro fertilization singletons: a systematic review and meta-analyses. *Eur J Obstet Gynecol Reprod Biol* 2009;**146**:(2): 138-148.

Mongelli M,Gardosi J. Longitudinal study of fetal growth in subgroups of a low-risk population. *Ultrasound Obstet Gynecol* 1995;**6**:(5): 340-344.

Mook-Kanamori DO, Steegers EA, Eilers PH, Raat H, Hofman A,Jaddoe VW. Risk factors and outcomes associated with first-trimester fetal growth restriction. *Jama* 2010;**303**:(6): 527-534.

Nelissen EC, Van Montfoort AP, Coonen E, Derhaag JG, Geraedts JP, Smits LJ, Land JA, Evers JL,Dumoulin JC. Further evidence that culture media affect perinatal outcome: findings after transfer of fresh and cryopreserved embryos. *Hum Reprod* 2012.

Nelissen EC, van Montfoort AP, Dumoulin JC,Evers JL. Epigenetics and the placenta. *Hum Reprod Update* 2011;**17**:(3): 397-417.

NICE,guidelines c (2008). Antenatal Care: Routine care for the healthy pregnant woman. National Collaborating Centre for Women's and Children's Health (UK)

Ong CY, Liao AW, Spencer K, Munim S,Nicolaides KH. First trimester maternal serum free beta human chorionic gonadotrophin and pregnancy associated plasma protein A as predictors of pregnancy complications. *Bjog* 2000;**107**:(10): 1265-1270.

Orasanu B, Jackson KV, Hornstein MD,Racowsky C. Effects of culture medium on HCG concentrations and their value in predicting successful IVF outcome. *Reprod Biomed Online* 2006;**12**:(5): 590-598.

Papaioannou GI, Syngelaki A, Poon LC, Ross JA,Nicolaides KH. Normal ranges of embryonic length, embryonic heart rate, gestational sac diameter and yolk sac diameter at 6-10 weeks. *Fetal Diagn Ther* 2010;**28**:(4): 207-219.

Poon LC, Karagiannis G, Staboulidou I, Shafiei A,Nicolaides KH. Reference range of birth weight with gestation and first-trimester prediction of small-for-gestation neonates. *Prenat Diagn* 2011;**31**:(1): 58-65.

Rivera RM, Stein P, Weaver JR, Mager J, Schultz RM,Bartolomei MS. Manipulations of mouse embryos prior to implantation result in aberrant expression of imprinted genes on day 9.5 of development. *Hum Mol Genet* 2008;**17**:(1): 1-14.

Romundstad LB, Romundstad PR, Sunde A, von During V, Skjaerven R,Vatten LJ. Increased risk of placenta previa in pregnancies following IVF/ICSI; a comparison of ART and non-ART pregnancies in the same mother. *Hum Reprod* 2006;**21**:(9): 2353-2358.

Rosenberg TJ, Garbers S, Lipkind H,Chiasson MA. Maternal obesity and diabetes as risk factors for adverse pregnancy outcomes: differences among 4 racial/ethnic groups. *Am J Public Health* 2005;**95**:(9): 1545-1551.

Rumball CW, Bloomfield FH, Oliver MH,Harding JE. Different periods of periconceptional undernutrition have different effects on growth, metabolic and endocrine status in fetal sheep. *Pediatr Res* 2009;**66**:(6): 605-613.

Saltvedt S, Almstrom H, Kublickas M, Reilly M, Valentin L,Grunewald C. Ultrasound dating at 12-14 or 15-20 weeks of gestation? A prospective cross-validation of established dating formulae in a population of in-vitro fertilized pregnancies randomized to early or late dating scan. *Ultrasound Obstet Gynecol* 2004;**24**:(1): 42-50.

Schielen PCJI, Elvers LH,Loeber JG (2003). Down syndroom risicoschatting in het eerste trimester door bepaling van PAPP-A en vrij b-hCG in serum. RIVM rapport 199101008/2003.

Ticconi C, Zicari A, Belmonte A, Realacci M, Rao Ch V,Piccione E. Pregnancy-promoting actions of HCG in human myometrium and fetal membranes. *Placenta* 2007;**28 Suppl A**: S137-143.

Turan N, Katari S, Gerson LF, Chalian R, Foster MW, Gaughan JP, Coutifaris C,Sapienza C. Inter- and intra-individual variation in allele-specific DNA methylation and gene expression in children conceived using assisted reproductive technology. *PLoS Genet* 2010;**6**:(7): e1001033.

van Montfoort AP, Hanssen LL, de Sutter P, Viville S, Geraedts JP,de Boer P. Assisted reproduction treatment and epigenetic inheritance. *Hum Reprod Update* 2012;**18**:(2): 171-197.

Verburg BO, Mulder PG, Hofman A, Jaddoe VW, Witteman JC,Steegers EA. Intra- and interobserver reproducibility study of early fetal growth parameters. *Prenat Diagn* 2008;**28**:(4): 323-331.

Watkins AJ, Platt D, Papenbrock T, Wilkins A, Eckert JJ, Kwong WY, Osmond C, Hanson M,Fleming TP. Mouse embryo culture induces changes in postnatal phenotype including raised systolic blood pressure. *Proc Natl Acad Sci U S A* 2007;**104**:(13): 5449-5454.

Watkins AJ, Ursell E, Panton R, Papenbrock T, Hollis L, Cunningham C, Wilkins A, Perry VH, Sheth B, Kwong WY*, et al.* Adaptive responses by mouse early embryos to maternal diet protect fetal growth but predispose to adult onset disease. *Biol Reprod* 2008;**78**:(2): 299-306.

Part II

Placental epigenetics

Chapter 4

Epigenetics and the placenta

Nelissen ECM
van Montfoort APA van
Dumoulin JCM
Evers JLH

Human Reproduction Update. 2011;17(3):397-417.Review.

Abstract

Background: The placenta is of utmost importance for the intrauterine fetal development and growth. Deregulation of placentation can lead to adverse outcomes for both mother and fetus, e.g. gestational trophoblastic disease (GTD), pre-eclampsia and fetal growth retardation. A significant factor in placental development and function is epigenetic regulation.

Methods: This review summarizes the current knowledge in the field of epigenetics in relation to placental development and function. Relevant studies were identified by searching PubMed, Medline and reference sections of all relevant studies and reviews.

Results: Epigenetic regulation of the placenta evolves during preimplantation development and further gestation. Epigenetic marks, like DNA methylation, histone modifications and non-coding RNAs, affect gene expression patterns. These expression patterns, including the important parent-of-origin-dependent gene expression resulting from genomic imprinting, play a pivotal role in proper fetal and placental development. Disturbed placental epigenetics has been demonstrated in cases of intrauterine growth retardation and small for gestational age, and also appears to be involved in the pathogenesis of pre-eclampsia and GTD. Several environmental effects have been investigated so far, e.g. ethanol, oxygen tension as well as the effect of several aspects of assisted reproduction technologies on placental epigenetics.

Conclusions: Studies in both animals and humans have made it increasingly clear that proper epigenetic regulation of both imprinted and non-imprinted genes is important in placental development. Its disturbance, which can be caused by various environmental factors, can lead to abnormal placental development and function with possible consequences for maternal morbidity, fetal development and disease susceptibility in later life.

Introduction

From its earliest stages till the end of pregnancy, the placenta is of paramount importance for the intrauterine development and growth of the fetus. It is responsible for the establishment of a tight contact between mother and conceptus, enabling the exchange of gas, nutrients and waste products. The placenta protects the fetus from maternal immune system attacks and secretes pregnancy-associated hormones and growth factors (Rossant *et al.*, 2001).

Deregulation of placentation can be detrimental to both mother and child; derailed growth can lead to invasive, sometimes malignant trophoblast disease in the mother (Hui *et al.*, 2005), whereas hypoplastic development is associated with maternal hypertension (pre-eclampsia) (Cross, 2003; Fisher, 2004) and fetal growth retardation (Chaddha *et al.*, 2004; Gluckman *et al.*, 2004; Monk *et al.*, 2004). Intra-uterine growth retardation and low birthweight (LBW) are major negative health predictors for newborns and children, and are associated with chronic diseases expressed later in life e.g. cardiovascular disease, hypertension and type 2 diabetes (Barker *et al.*, 1993; Curhan *et al.*, 1996a; Curhan *et al.*, 1996b; Boyko, 2000; Eriksson *et al.*, 2003; Ross *et al.*, 2008). A significant factor in placental development and function is epigenetic regulation. Imprinted genes for instance, which are epigenetically regulated, are abundantly expressed in the placenta and usually lacking in non-placental organisms (Reik *et al.*, 2001b).

In the present review, we discuss the role of epigenetics in placental development and outline the progress in both animal and human research in recent years. Our intention is to offer the clinician insight into the consequences of disturbed placental epigenetics and to focus on environmental effects which may cause these disturbances.

Methods

To generate this review, a thorough literature search was repeatedly made in PubMed and Medline, with a limitation for articles written in English language. Search terms used were placenta, epigenetic, DNA methylation, histone modification, non-coding RNA, imprinting, environment. In addition, reference sections of all relevant studies or reviews were manually searched for more information.

Epigenetics

Although the cells in a human body contain the same DNA sequence, the function and phenotype differ (Reik, 2007). This implies that, apart from genetic programming, the phenotype is regulated by another phenomenon. This is called *epi*-(Greek for upon, above)-*genetics*. Epigenetics is defined as the study of changes in phenotype or gene expression caused by mechanisms other than changes in the underlying DNA sequence. The usage of the word in scientific discourse is more narrow, referring to changes in gene function which are heritable (over rounds of cell division and sometimes transgenerationally) (Russo *et al.*, 1996). During development, differentiated cells accumulate epigenetic modifications (or marks) that differ from those of pluripotent cells, and differentiated cells of different lineages also accumulate different marks (Reik, 2007). Certain epigenetic marks can be removed before a cell divides or within very few cell divisions (short-term flexibility), while other marks can be maintained for many divisions (long-term stability and heritability). Under the definition which strictly requires heritability, alterations that last less than one cell cycle do not qualify as epigenetic. Bird recently discussed the restrictiveness of this heritable view and redefined epigenetics as "the structural adaption of chromosomal regions to register, signal or perpetuate altered activity states" (Bird, 2007). This definition focuses not only on genes but also on chromosomes without the constraint of heritability.

Epigenetic marks, deposited early in development, are able to adapt themselves throughout life in response to intrinsic and environmental stimuli. Accordingly, epigenetic disturbance may lead to different phenotypes and diseases like cancer. Epigenetic regulation controls transcription at three levels, which are also displayed in the placenta: DNA (DNA methylation), protein (histone modifications) and RNA (non-coding RNAs).

DNA methylation

DNA methylation is the best-characterized epigenetic modification. Enzymes called DNA methyltransferases (DNMTs) catalyse the addition of a methyl group to the cytosine ring to form methyl cytosine, using S-adenosylmethionine as a methyl donor (Herman *et al.*, 2003). After DNA replication, DNMT1 is the predominant mammalian DNA methylating enzyme responsible for the restoration of hemi-methylated sites to full methylation, called maintenance methylation. DNMT3A and DNMT3B are mainly involved in the methylation

of new sites, called *de novo* methylation (Laird, 2003). DNMT3L is postulated to play a regulatory role in DNA methylation without DNA methyltransferase activity in itself (Okano *et al.*, 1998). In humans and other mammals, the post-replicative DNA modification occurs predominantly on cytosines that precede a guanosine in the DNA sequence (the CpG dinucleotide) (Herman *et al.*, 2003). These dinucleotides can be clustered in small stretches of DNA termed CpG islands, which are often associated with promoter regions. Most CpG sites outside of CpG islands are methylated suggesting a role in the global maintenance of the genome, while most CpG islands in gene promoters are unmethylated which allows active gene transcription (Herman *et al.*, 2003; Weber *et al.*, 2007). The importance of DNA methylation for gene expression, and especially for transcriptional silencing, is apparent in the exceptions to the rule that CpG islands associated with gene promoter regions are unmethylated (Bird, 2002). These exceptions are for instance the fully methylated CpG islands associated with the silenced alleles of some imprinted genes (see below) or on the inactive X chromosome.

DNA methylation in the placenta

In mammalian preimplantation development, DNA methylation of non-imprinted genes is subject to a global reprogramming process after fertilization, with phases of active and passive demethylation followed by *de novo* methylation (Mayer *et al.*, 2000; Horsthemke *et al.*, 2005). This *de novo* methylation occurs by the blastocyst stage, but is restricted to the inner cell mass (ICM), whereas the trophectoderm (TE) is virtually lacking methylation (Santos *et al.*, 2002). An inequality is thus established between both cell lineages at the time of differentiation into embryonic and extraembryonic lineages (Santos *et al.*, 2002). This epigenetic inequality with higher overall DNA methylation levels in the embryo compared with the placenta is maintained throughout gestation (Santos *et al.*, 2002). Earlier investigations have also shown that hypomethylation is maintained in all derivates of the extraembryonic lineage (Chapman *et al.*, 1984; Rossant *et al.*, 1986). At term, two regulating domains in the IGF2-H19 region are for instance hypomethylated in placenta, while in neonatal blood these regions are methylated on one of the two alleles (Guo *et al.*, 2008). Despite the global hypomethylation of the trophoblast lineage, DNA methylation is indispensable for normal development of extraembryonic tissues, especially for the invasive behaviour of trophoblast cells. Administration of a single dose

of 5'-aza-2'-deoxycytidine (a DNA methylation inhibitor) to pregnant rats at different stages of development, disrupts trophoblast proliferation (Vlahovic *et al.*, 1999; Serman *et al.*, 2007) and in human choriocarcinoma-derived cell lines 5'-aza-2'-deoxycytidine disrupts trophoblast migration (Rahnama *et al.*, 2006). Furthermore, knockout mice studies of DNA methyltransferases *Dnmt1* and *Dnmt3L* have shown that the placentas of these homozygous mice exhibit multiple morphological defects, like chorioallantoic fusion defects and lack of labyrinth formation (Li *et al.*, 1992; Bourc'his *et al.*, 2001; Arima *et al.*, 2006). These effects may be mediated by a loss of imprinting (LOI) (see below) since mutant *Dnmt1* resulted in biallelic expression of certain genes in the regulating domains *Igf2, Snrpn* and *Peg3*, while genes in the *Kcnq1* domain were less sensitive to absence of *Dnmt1* (Weaver *et al.*, 2010).

Placentation displays many similarities with tumourigenesis. Trophoblasts proliferate, migrate and invade the uterine wall and its vasculature and escape from immune detection, like cancer cells. Normal human cytotrophoblasts express functional tumour-associated genes, several of which are also essential for the development of certain malignancies (Ferretti *et al.*, 2007; Koslowski *et al.*, 2007). Recent observations showed epigenetic regulation by DNA methylation and histone modification of some tumour suppressor genes like Maspin, RASSF1A and APC in human placentas (Dokras *et al.*, 2006; Chiu *et al.*, 2007; Wong *et al.*, 2008). In addition, Novakovic *et al.* investigated the extent of tumour-associated methylation in first-trimester cytotrophoblasts and term placenta, and demonstrated a methylation-induced reduction in expression in a small subset of genes as part of normal human placentation (Novakovic *et al.*, 2008).

Besides a role of DNA methylation in placental morphology, it also affects placental physiology. For instance, the tightly controlled regulation of plasma concentrations of biologically active vitamin D is epigenetically uncoupled in pregnancy (Novakovic *et al.*, 2009). Biologically active vitamin D regulates calcium homeostasis, immunomodulation, cellular differentiation and apoptosis (DeLuca, 2004; Dusso *et al.*, 2005; Anderson *et al.*, 2006). In addition, vitamin D deficiency has been linked to placental insufficiency, e.g. pre-eclampsia (Bodnar *et al.*, 2007; Seely, 2007). Promoter methylation downregulates the promoter activity of vitamin D hydroxylase (CYP24A1) and abolishes vitamin D-mediated feedback activation (Novakovic *et al.*, 2009). This leads to significantly elevated levels of active vitamin D at the fetomaternal interface, suggesting a role in pregnancy progression (Novakovic *et al.*, 2009).

Histone modification

The nucleosome is, together with the genomic DNA, an important constituent of chromatin. The nucleosome is a protein complex, consisting of two copies of each of the four core histones (H2A, H2B, H3 and H4), around which the DNA is wrapped. The N-terminal histone tail protrudes from the chromatin. Epigenetic modifications at the N-terminal tail of nucleosomal histones involve methylation, acetylation, phosphorylation and ubiquitinylation of selected amino acids, that can impose either transcriptionally repressive or transcriptionally permissive chromatin structures (Turner, 2002; Kimura *et al.*, 2004). These modifications are accomplished by a range of enzymes including histone methyltransferases (HMTs), acetyltransferases (HATs), kinases and ubiquitylases, while histone demethylases (HDMs), deacetylases (HDACs), phosphatases and deubiquitylases are able to remove the mark from the histone tail. Proteins can recognize and bind to these specific modifications and exert an effect on gene activity. Polycomb Group (PcG) proteins for instance, which mutually form complexes, are known for their transcriptional repression (Niessen *et al.*, 2009). Furthermore, histone acetylation usually marks active genes as does di- or trimethylation of lysine residue 4 of histone H3 (H3K4me2, K4me3), whereas H3K9me2/3 and H3K27me3 constitute repressive marks (Peterson *et al.*, 2004; Sims *et al.*, 2006). Repressive histone modifications seem to confer short-term, flexible silencing which is important for developmental plasticity, whereas DNA methylation is believed to be a more stable, long-term silencing mechanism (Boyer *et al.*, 2006; Lee *et al.*, 2006; Reik, 2007).

Histone modifications in the placenta

Like DNA methylation, histone methylation also plays a role in cell differentiation at the blastocyst stage. H3 arginine methylation predisposes blastomeres to contribute to the pluripotent cells of the ICM, which appears to require higher global levels of H3 arginine methylation than the TE/trophoblast lineage (Torres-Padilla *et al.*, 2007). This epigenetic disparity, with higher histone modification levels in the embryonic lineage compared with the trophoblast lineage, is seen more globally. Besides in many mouse studies, this has also been described in other mammalian species: bovine, sheep and rabbit (Manes *et al.*, 1981; Santos *et al.*, 2003; Beaujean *et al.*, 2004). Nevertheless, as with DNA methylation, again these lower modification levels in the trophoblast lineage are indispensable for normal extraembryonic development. H3K27 methylation for instance,

is mediated by several protein aggregates, like PcG repressive complex (PRC) 2 containing PcG members and HDACs (Hemberger, 2007). Mutants of PcG members are characterized by failure of amnion and chorion formation (O'Carroll *et al.*, 2001; Pasini *et al.*, 2004) or failure of invasive trophoblast giant cell differentiation (Wang *et al.*, 2001; Wang *et al.*, 2002). The above described lineage-specific DNA methylation and histone modification levels can influence cell fate determination (Reik *et al.*, 2003b; Torres-Padilla, 2008) and emphasize the difference in regulatory mechanisms between embryonic and placental tissues.

Selective activation of placental-specific genes requires specific roles for histone modifying enzymes. The expression of the human transcription factor GCMa for instance, necessary for regulating syncytin which mediates proper trophoblastic fusion, depends on regulation by HDACs and HATs (Chuang *et al.*, 2006). Another example of histone modification-mediated placental-specific gene regulation concerns five genes of the human growth hormone (*hGH)* cluster. These are highly conserved in structure, yet are expressed selectively in the placenta or pituitary. Placental growth hormone gradually replaces pituitary growth hormone and these placental secretions appear to have important implications for physiological adjustment to gestation and especially in the control of maternal IGF1 levels (Alsat *et al.*, 1998). The transcriptional activation of the placental-specific and pituitary-specific hGH genes is differentially regulated with different roles for HATs and HMTs co-activator complexes in each of these tissues (Kimura *et al.*, 2004).

Non-coding RNAs

In the past years, it has been discovered that the majority of the mammalian genome is transcribed and that these transcripts mainly consist of non-coding (nc) RNAs (Okazaki *et al.*, 2002; Carninci *et al.*, 2005; Katayama *et al.*, 2005; Engstrom *et al.*, 2006). These ncRNAs can be classified according to their function or length. When ncRNAs act in *cis*, they are able to regulate the expression of one or more genes on the same chromosome. On the other hand when ncRNAs act in *trans*, they are able to regulate the expression of one or more genes on different chromosomes or regulate mature RNAs in the cytoplasm (Koerner *et al.*, 2009). *Cis*-acting functions have been associated with macro ncRNAs and *trans*-acting functions with short ncRNAs. Macro ncRNAs can be a few hundred to several hundred thousand nucleotides (nt) long. Examples of short ncRNAs

are short interfering (si) RNAs (21 nt), micro (mi) RNAs (~22 nt), piwi-interacting RNAs (26-31 nt) and short nucleolar (sno) RNAs (60-300 nt) (Koerner *et al.*, 2009). Well-studied mammalian macro ncRNAs are *Xist* (inactive X-specific transcript) and *Tsix* (X-specific transcript) which are involved in X-chromosome inactivation in female mammals.

ncRNAs in the placenta

In the placenta, repression of multiple genes in the *Igf2r* and *Kcnq1* clusters on the paternal chromosome depends on the macro ncRNAs *Airn* and *Kcnq1ot1* by an unknown mechanism (Sleutels *et al.*, 2002; Mancini-Dinardo *et al.*, 2006; Shin *et al.*, 2008). There are transcription-based silencing models that do not require the ncRNA product itself, but only its transcription which interferes for instance with *cis*-regulatory activator elements or the activation of *cis*-regulatory silencing elements. On the other hand in ncRNA-based silencing models, ncRNAs coat the region containing silenced genes and recruit silencing factors (Pauler *et al.*, 2007). Recently, two studies found evidence for the ncRNA-based silencing model concerning the macro ncRNAs *Airn* and *Kcnq1ot1*. They indicated that *Airn* and *Kcnq1ot1* are themselves directly involved in the repression of placental genes (Pandey *et al.*, 2008; Terranova *et al.*, 2008). For the involvement of ncRNAs in placental gene clusters with parent-of-origin-expression, see section on genomic imprinting.

Additionally, many epigenetic modifiers interact with one another. For example, the acquisition of histone modifications and DNA methylation is interdependent. They interplay with each other and also with regulatory proteins and non-coding RNAs (Lehnertz *et al.*, 2003; Esteve *et al.*, 2006; Vire *et al.*, 2006; Januchowski *et al.*, 2007; Delcuve *et al.*, 2009). In the placenta, for instance the ncRNA *Kcnq1ot1* localizes to chromatin and recruits repressive histone marks to the whole imprinted cluster (Pandey *et al.*, 2008). Furthermore, imprinted macro ncRNAs can serve as host transcripts for *trans*-acting short ncRNAs, which suggests a functional link between the two (Koerner *et al.*, 2009).

Genomic imprinting

Following experiments with maternal and paternal pronuclei, it was discovered that both parental genomes are required in the embryo for viable development (Barton *et al.*, 1984; McGrath *et al.*, 1984; Surani *et al.*, 1984). Imprinted genes are defined by the functional non-equivalence of the maternal and paternal

copy resulting in monoallelic expression in a parent-of-origin dependent manner. During the process of imprinting, the male and female germ line confer a sex-specific mark (imprint) on certain chromosomal regions (Reik *et al.*, 2001b). Only one allele of the imprinted genes, the maternal or paternal, can be active and expressed. Note that the imprints themselves can be associated with activity or inactivity (Ferguson-Smith *et al.*, 2006). This active or inactive allele is epigenetically marked by the earlier described histone modifications, DNA methylation or both (Reik *et al.*, 2001a). Imprinted genes are not randomly distributed in the genome, but tend to occur predominantly in clusters. Each cluster is controlled by an imprinting control region (ICR) which usually contains a stably maintained or developmentally changing Differentially Methylated Region (DMR) (Wood *et al.*, 2006; Edwards *et al.*, 2007). The existence of these ICRs suggests that the primary control of imprinting is not at a single gene level, but at chromosomal level (Buiting *et al.*, 1995). Furthermore, imprinting (parent-of-origin-specific gene transcription) is tissue-specific, species-dependent and developmentally regulated (Morison *et al.*, 1998; Fowden *et al.*, 2006; Monk *et al.*, 2006). Besides assuming imprinting as a simple pattern resulting in either paternal or maternal expression, more complex and diverse patterns of effect have been found (Wolf *et al.*, 2008). The quantitative effect of an imprinted allele not only depends on its parent-of-origin, but also on the allele it is paired with at a locus (Wolf *et al.*, 2008).

In addition to allele-specific methylation and histone modifications, imprinting is regulated by non-coding RNAs (Spahn *et al.*, 2003). The macro ncRNAs *Airn, Kcnq1ot1 and Nespas* are involved in genomic imprinting control (Koerner *et al.*, 2009). Each imprinted gene cluster, which contains an ICR, expresses at least one macro ncRNA gene (Edwards *et al.*, 2007; Royo *et al.*, 2008). An unmethylated ICR is required for imprinted macro ncRNA expression and the macro ncRNA is generally expressed from the other chromosome than most imprinted mRNA genes (Koerner *et al.*, 2009). For instance, the imprinted Igf2r cluster contains three maternally expressed genes [*Igf2r, Slc22a2* (solute carrier family 22,a2) and *Slc22a3*]. The paternal unmethylated ICR expresses a macro ncRNA named *Airn*, which silences paternal *Igf2r, Slc22a2* and *Slc22a3* expression (Royo *et al.*, 2008). The mechanisms by which these ncRNAs are responsible for the induced repression in imprinted loci are still not well characterized.

After fertilization, a genome-wide DNA demethylation and remethylation takes place in the embryo (Mayer *et al.*, 2000; Horsthemke *et al.*,

2005). However, imprinted genes escape this epigenetic reprogramming (Mayer *et al.*, 2000). They are protected from demethylation because it is important that the parental imprints are preserved in the developing embryo (Tremblay *et al.*, 1995). Later in fetal development, when the germ cells initially enter the gonad, genomic imprints are erased. They are newly established during later stages of gametogenesis (paternal imprints in spermatozoa and maternal imprints in oocytes) and maintained during post-zygotic development (Lucifero *et al.*, 2004a; Swales *et al.*, 2005). Both Dnmt3a and Dnmt3L are at least required for methylation of most imprinted loci in germ cells and are therefore implicated in maternal and paternal imprinting (Hata *et al.*, 2002; Kaneda *et al.*, 2004). In contrast, Miri and Varmuza recently proposed the 'Mother Knows Best' model of genomic imprinting. Evidence is accumulating from both wild-type and manipulated mammalian embryos indicating that genomic imprinting is a maternal effect regulated by oocyte proteins, and acting in part during the long first cell cycle that precedes cleavage divisions (Miri *et al.*, 2009). In this proposed model, the oocyte contains remodelling components which are necessary for paternal genome epigenetic establishment just after fertilization (Miri et al 2009). In the placenta, maintenance of imprinting has been recently inferred to depend more on repressive histone methylation and ncRNAs than on DNA methylation (Wagschal *et al.*, 2006; Wagschal *et al.*, 2008). In fact, placenta-specific imprinting, in contrast to the embryo, along mouse distal chromosome 7 is largely independent of DNA methylation in its maintenance (Tanaka *et al.*, 1999; Lewis *et al.*, 2004; Umlauf *et al.*, 2004).

Genomic imprinting arose during mammalian evolution (around 150 million years ago) and might be associated with the evolution of intrauterine development that requires formation of a placenta (Reik *et al.*, 2001b). Therefore, many researchers have speculated that some aspect of placentation benefits from genomic imprinting (Coan *et al.*, 2005; Wagschal *et al.*, 2006; Charalambous *et al.*, 2007; Renfree *et al.*, 2008). There are several theories about the purpose of genomic imprinting. One hypothesis is that genomic imprinting is a by-product of a self-defence mechanism against exogenous DNAs or retrotransposons without a meaning in itself (Barlow, 1993; Yoder *et al.*, 1997). Accumulating evidence describes the central role of DNA methylation in repression of retroviruses and retrotransposons, as well as genomic imprinting (Yoder *et al.*, 1997; Okano *et al.*, 1999; Bourc'his *et al.*, 2001; Hata *et al.*, 2002; Bourc'his *et al.*, 2004; Kaneda *et al.*, 2004). Also, the mammalian genome contains many

retrotransposon-derived sequences compared with the genome of other higher vertebrates. It has been suggested that this phenomenon has evolved over time but this does not explain why not all imprinted genes are methylated.

Another hypothesis is based on parthenogenetic placentation, or the ovarian time bomb hypothesis (Varmuza *et al.*, 1994; Weisstein *et al.*, 2002). Parthenotes (two maternal genomes) have well-developed small embryos which develop until early post-implantation stages, but they have almost non-existent trophoblast. Therefore, it is hypothesized that female mammals are protected from malignant ovarian trophoblastic disease. Viable offspring can be generated from mice oocytes where one of the two maternal haploid genomes has a deletion in the paternally imprinted H19 ICR (Kono *et al.*, 2004). This illustrates that paternal imprinting prevents parthenogenetic development in mice. Therefore, genomic imprinting is a barrier to parthenogenesis, which can protect female mammals from malignant trophoblastic disease. They also demonstrated that appropriate expression of *Igf2* and *H19* genes is extremely important for normal development (Kono *et al.*, 2004). This theory does not explain why genes which are not involved in placental development are still imprinted.

Furthermore, the 'complementation hypothesis' has been proposed. It explains the origin of imprinting arguing that it is essential for mammalian development as a mechanism regulating complementary expression profiles of paternal and maternal genomes because both cannot be expressed from the same chromosomes simultaneously, even when the parental imprints are completely erased (Lee *et al.*, 2002; Kaneko-Ishino *et al.*, 2003). This hypothesis uses the mechanism of complementation under the selective pressure predicted by the genetic conflict hypothesis.

Most popular is the conflict or 'battle of the sexes' theory (Moore *et al.*, 1991; Haig, 2004). Following this theory, the father wants maximal growth and good health for his offspring to ensure successful survival against competition allowing the paternal genome to be passed on to successive generations. The mother, however, despite wanting the same outcome, also wants to be able to reproduce again and limit the drain on her resources to maintain her own health. This competition within the embryo and/or placenta between paternal genes attempting to enhance growth and maternal genes trying to limit growth is reflected in either intrauterine growth restriction (IUGR) or overgrowth in imprinting disorder diseases. The conflict hypothesis has been challenged by

the lack of phenotype observed in knockout mice of the maternally imprinted *Snrpn* gene (Yang *et al.*, 1998) and the growth inhibitory rather than enhancer effect of the paternally expressed *ZAC* gene (Piras *et al.*, 2000).

Imprinting controlling placental and fetal development

The importance of imprinted genes for the placenta is underlined by the many imprinted genes that are expressed in the placenta (Reik *et al.*, 2001b; Ferguson-Smith *et al.*, 2006; Tycko, 2006; Hemberger, 2007), the distinct imprinted gene expression profile displayed in the placenta and embryo as compared with adult tissues (Steinhoff *et al.*, 2009) and the fact that non-placental organisms like fish, reptiles and chickens lack imprinting (Yokomine *et al.*, 2001; Reik *et al.*, 2003a; Yokomine *et al.*, 2005).

Why is the placenta such an important site of imprinted gene action? Knockout (loss of expression) and transgenic (over-expression) data of placental imprinted genes have revealed a range of (aberrant) phenotypic patterns. Imprinted genes, such as *Ascl2, Phlda2 and Peg10* (a newly discovered retrotransposon-derived gene) appear to be indispensable for proper placental morphology and function, while others, like *Igf2,* are involved in nutrient supply regulation (Guillemot *et al.*, 1994; Salas *et al.*, 2004; Angiolini *et al.*, 2006; Ono *et al.*, 2006). The latter was found to be a major modulator of placental and fetal growth, as it regulates the development of the diffusional permeability capacity in the placenta (Constancia *et al.*, 2002; Sibley *et al.*, 2004). Recently, it has been demonstrated in the mouse that placental phenotype, like morphology and diffusional exchange characteristics, depends on the degree of *Igf2* gene ablation and the interplay between placental and fetal *Igf2* (Coan *et al.*, 2008). As described before, a number of genes are imprinted specifically in the placenta, but genes imprinted in both the embryo and the placenta can also be essential for placental development (Wagschal *et al.*, 2006).

To date, more than 130 imprinted genes in mice and at least 70 in humans have been discovered (http://www.geneimprint.com, www.mgu.har.mrc.ac.uk, http://igc.otago.ac.nz), of which several are imprinted specifically in the placenta. There is an extensive conservation between mouse and human genes that are imprinted in both the embryo and extraembryonic tissues. Conserved maternal expression was found for the imprinted genes *PHLDA2, SLC22A18, SLC22A1LS* and *CDKN1C* (Monk *et al.*, 2006). Genes from the *IGF2R* domain showed human placental imprinting in only a minority of the samples

(Monk *et al.*, 2006). However, a lack of imprinting was demonstrated in the human *KCNQ1* domain and the human orthologues of the mouse placental-specific imprinted genes *Gatm* and *Dcn*. Although maternal expression was observed in the mouse, human expression appeared largely bi-allelic from first-trimester trophoblast till term (Monk *et al.*, 2006).

Although imprinting in human placentas has been increasingly studied, the parental origin of the allele-specific expression of several human placental imprinted genes is still unknown (Table I). Besides, placental imprinting of these genes is a dynamic process which evolves during human pregnancy (Pozharny *et al.*, 2010). Especially in first-trimester placentas, several genes, in contrast to *IGF2*, displayed a significantly higher rate of LOI than term placentas, although little difference in gene expression was seen (Pozharny *et al.*, 2010). Among these genes is *H19* with, before 10 weeks of pregnancy, bi-allelic expression in 28% of the placental tissues. After 10 weeks, only mono-allelic expression was measured (Yu *et al.*, 2009). These investigations demonstrate that the parent-of-origin effect is not established completely in the first trimester of human pregnancy. Instead, placental imprinting is a dynamic, maturational process. It is postulated that these developmental changes in LOI, without necessarily affecting the gene expression, play an important role in placental maturation and development (Pozharny *et al.*, 2010). Also in term placentas, the mono-allelic expression of imprinted genes is not always very strict. Lambertini *et al.* (2008) for instance analysed the expression of nine imprinted genes and found that in apparently normal term placentas several genes showed bi-allelic expression in some but not all placentas (Lambertini *et al.*, 2008). For *ZAC/PLAGL1,* the expression of the maternally imprinted allele accounted for only 1-3% of the total expression of this gene. On the other hand, the maternally imprinted *IGF2* allele could account for 20-50% of the expression and the maternally imprinted *PEG3* sometimes for even more than 50%. Interestingly, *H19* and *DLK1* never showed LOI (Lambertini *et al.*, 2008). This has also been reported in mice. In a few placentas about 20% LOI was reported for *Snrpn* while, just as in human, *H19* escaped LOI (Fortier *et al.*, 2008). There are also imprinted genes that display mono-allelic expression just in first trimester placental tissues, like *CTNNA3* (Oudejans *et al.*, 2004; van Dijk *et al.*, 2004). The parent-of-origin effect of this gene is also trophoblast cell type-dependent: bi-allelic expression in extravillous trophoblast, maternal expression in villous cytotrophoblast and expression is lost following epithelial-mesenchymal transition (van Dijk

et al., 2004). This trophoblast cell type-dependent imprinting of *CTNNA3* is identical to *CDKN1C* imprinting with respect to trophoblast cell type (villous) and parental origin of the expressed allele (maternal). Therefore, gene dosage compensation of both *CTNNA3* and *CDKN1C* in the placenta is suggested to share a conserved regulatory mechanism correlating with an early step in trophoblast determination, i.e. differentiation into villous or extravillous trophoblast (van Dijk *et al.*, 2004).

Paternally expressed genes tend to enhance fetal growth while maternally expressed genes tend to suppress it (Fowden *et al.*, 2006). Knockout studies of several paternally or maternally imprinted genes result in IUGR and smaller placental size or overgrowth and hyperplasia of placenta, respectively (Lefebvre *et al.*, 1998; Takahashi *et al.*, 2000; Frank *et al.*, 2002). Furthermore, certain maternal genes are required for proper development of the embryo, whereas extraembryonic tissues depend on the presence of active paternal genes. An excess of paternally derived chromosomes leads to a complete (no maternal genome) or partial (lower amount of maternal chromosomes) mole (Devriendt, 2005). Accordingly, hydatidiform moles are characterized by a reduced or even lack of embryonic development and excessive trophoblastic proliferation.

Disturbed placental epigenetics

Several researchers examined the role of epigenetic disturbance in human placental-related pathologies like small for gestational age (SGA), IUGR, pre-eclampsia and gestational trophoblastic disease (GTD) (Table II).

Many cases of IUGR, in which a fetus is not able to achieve its genetically determined potential size, are idiopathic and utero-placental insufficiency is regarded as an important factor in these cases. SGA infants are smaller than the 10th percentile for the gestational age of pregnancy. It is clinically important to distinguish fetal constitutional smallness from fetal growth failure to ensure appropriate medical care, prevent unnecessary elective Caesarean sections and premature births. An IUGR infant may or may not be SGA but it always implies a pathological process like an underlying utero-placental insufficiency (Bamberg *et al.*, 2004; Maulik, 2006). Therefore, further research is conducted to discover possible biomarkers for IUGR and to elucidate the aetiology of IUGR.

Table I Human placental imprinted genes

Gene	Name	Location	References	Placental tissue	Mono-allelic expression	Parental expression in placenta			Parental expression (not placental-specific) [1]
						Paternal	Maternal	Not analysed	
DLK1	delta-like 1 homolog	14q32	Pozharny *et al.* 2010	1st-trimester	minor subset	x			P
			Lambertini et al. 2008, Pozharny *et al.* 2010	term	yes	x			P
IGF2	insulin-like growth factor 2 (somatomedin A)	11p15.5	Pozharny *et al.* 2010	1st-trimester	subset	x			P
			Apostolidou *et al.* 2007, Lambertini *et al.* 2008	term	subset	x			P
MEST/PEG1 isoform 1	mesoderm specific transcript homolog	7q32	Pozharny *et al.* 2010	1st-trimester	subset	x			P
			Apostolidou *et al.* 2007, Lambertini *et al.* 2008	term	subset	x			P
MEST/PEG1 isoform 2	mesoderm specific transcript homolog	7q32	McMinn *et al.* 2006	3rd-trimester/ term	large subset	x			P
PEG3	paternally expressed 3	19q13.4	Pozharny *et al.* 2010	1st-trimester	subset	x			P
			Lambertini et al. 2008, Pozharny *et al.* 2010	term	subset	x			P
PEG10	paternally expressed 10	7q21	Pozharny *et al.* 2010	1st-trimester	subset	x			P
			Lambertini *et al.* 2008	term	subset	x			P

Table I continued

Gene	Name	Location	References	Placental tissue	Mono-allelic expression	Parental expression in placenta			Parental expression (not placental-specific) [1]
						Paternal	Maternal	Not analysed	
SNRPN	small nuclear ribonucleoprotein polypeptide N	15q11.2	Pozharny *et al.* 2010	1st-trimester	minor subset	x			P
			Diplas et al. 2009, Pozharny *et al.* 2010	term	yes	x			P
PLAGL1/ZAC	pleiomorphic adenoma gene-like 1	6q24-q25	Pozharny *et al.* 2010	1st-trimester	minor subset	x			P
			Lambertini et al. 2008, Pozharny *et al.* 2010	term	yes	x			P
CDKN1C	cyclin-dependent kinase inhibitor 1C (p57)	11p15.5	Monk *et al.* 2006	term	yes		x		M
H19	H19, imprinted maternally expressed transcript (non-protein coding)	11p15.5	Pozharny *et al.* 2010, Yu *et al.* 2009	1st-trimester	subset		x		M
			Lambertini *et al.* 2008, Yu *et al.* 2009	term	yes		x		M
MEG3	maternally expressed 3 (non-protein coding)	14q32	Pozharny *et al.* 2010	1st-trimester	minor subset		x		M
			Lambertini *et al.* 2008, Pozharny *et al.* 2010	term	yes		x		M
PHLDA2	pleckstrin homology-like domain, family A, member 2	11p15.5	Pozharny *et al.* 2010	1st-trimester	minor subset		x		M

Table I continued

Gene	Name	Location	References	Placental tissue	Mono-allelic expression	Parental expression in placenta			Parental expression (not placental-specific) [1]
						Paternal	Maternal	Not analysed	
			Apostolidou *et al.* 2007, Monk *et al.* 2006, Diplas *et al.* 2009	term	yes		x		M
SLC22A18	solute carrier family 22, member 18	11p15.5	Pozharny *et al.* 2010	1st-trimester	subset		x		M
			Monk *et al.* 2006, Diplas *et al.* 2009, Pozharny *et al.* 2010	term	subset		x		M
SLC22A18AS/ SLC22A1LS	solute carrier family 22 (organic cation transporter), member 18 antisense	11p15.5	Monk *et al.* 2006	term	yes		x		M
TP73	tumor protein p73	1p36.3	Pozharny *et al.* 2010	1st-trimester	subset		x		M
			Lambertini et al. 2008, Pozharny *et al.* 2010	term	large subset		x		M
CD44	CD44 molecule (Indian blood group)	11p13	Pozharny *et al.* 2010	1st-trimester	minor subset		postulated		postulated: M
			Diplas *et al.* 2009, Pozharny *et al.* 2010	term	yes		postulated		postulated: M
CTNNA3	catenin (cadherin-associated protein), alpha 3	10q22.2	Oudejans *et al.* 2004, Van Dijk *et al.* 2004	1st-trimester	subset		x		provisional data: M
			Diplas *et al.* 2009	term	yes			x	provisional data: M

Table I continued

Gene	Name	Location	References	Placental tissue	Mono-allelic expression	Parental expression in placenta			Parental expression (not placental-specific) [1]
						Paternal	Maternal	Not analysed	
CTNND2	catenin, delta 2 (neural plakophilin-related arm-repeat protein)	5p15.2	Diplas *et al.* 2009	term	yes			x	no record
CYR61	cysteine-rich, angiogenic inducer, 61	1p31-p22	Diplas *et al.* 2009	term	yes			x	no record
DLX5	distal-less homeobox 5	7q22	Diplas *et al.* 2009	term	yes			x	M
EPS15	epidermal growth factor receptor pathway substrate 15	1p32	Pozharny *et al.* 2010	1st-trimester	minor subset		postulated		no record
			Diplas *et al.* 2009, Pozharny *et al.* 2010	term	yes		postulated		no record
GDNF	glial cell derived neurotrophic factor	5p13.1-p12	Diplas *et al.* 2009	term	yes			x	no record
HYMAI	hydatidiform mole associated and imprinted (non-protein coding)	6q24.2	Diplas *et al.* 2009	term	yes			x	P
IL1B	interleukin 1, beta	2q14	Diplas *et al.* 2009	term	yes			x	no record
INS	insulin	11p15.5	Diplas *et al.* 2009	term	yes			x	P
KCNQ1OT1/ LIT1	KCNQ1 overlapping transcript 1 (non-protein coding)	11p15	Monk *et al.*2006	1st-trimester	minor subset	x			P
			Monk *et al.* 2006	term	no				P

Table I continued

Gene	Name	Location	References	Placental tissue	Mono-allelic expression	Parental expression in placenta			Parental expression (not placental-specific) [1]
						Paternal	Maternal	Not analysed	
			Guo *et al.* 2008	3rd-trimester/ term	yes	x			P
MAGEL2	MAGE-like 2	15q11-q12	Diplas *et al.* 2009	term	yes			x	P
MAGI2	membrane associated guanylate kinase, WW and PDZ domain containing 2	7pq21	Diplas *et al.* 2009	term	yes			x	predicted: M
MAPK12	mitogen-activated protein kinase 12	22q13.33	Diplas *et al.* 2009	term	yes			x	no record
MESTIT1	MEST intronic transcript 1 (non-protein coding)	7q32	Diplas *et al.* 2009	term	yes			x	P
MKRN3	makorin ring finger protein 3	15q11-q13	Diplas *et al.* 2009	term	yes			x	P
NDN	necdin homolog	15q11.2-q12	Diplas *et al.* 2009	term	yes			x	P
NGF	nerve growth factor (beta polypeptide)	1p13.1	Diplas *et al.* 2009	term	yes			x	no record
NNAT	neuronatin	20q11.2-q12	Diplas *et al.* 2009	term	yes			x	P
PCNA	proliferating cell nuclear antigen	20pter-p12	Diplas *et al.* 2009	term	yes			x	no record
SDHD	succinate dehydrogenase complex, subunit D, integral membrane protein	11q23	Diplas *et al.* 2009	term	yes			x	conflicting data

Table I continued

Gene	Name	Location	References	Placental tissue	Mono-allelic expression	Parental expression in placenta			Parental expression (not placental-specific) [1]
						Paternal	Maternal	Not analysed	
SLC22A3	solute carrier family 22 (extraneuronal monoamine transporter), member 3	6q26-q27	Monk *et al.*2006	1st-trimester	yes			x	M
SGCE	sarcoglycan, epsilon	7q21-q22	Diplas *et al.* 2009	term	yes			x	P
SNORD64	small nucleolar RNA, C/D box 64	15q12	Diplas *et al.* 2009	term	yes			x	P
SNORD108	small nucleolar RNA, C/D box 108	15q11.2	Diplas *et al.* 2009	term	yes			x	P
SNURF	SNRPN upstream reading frame	15q12	Diplas *et al.* 2009	term	yes			x	P
UBE3A	ubiquitin protein ligase E3A	15q11-q13	Diplas *et al.* 2009	term	yes			x	M
WT1	Wilms tumor 1	11p13	Diplas *et al.* 2009	term	yes			x	P
			Jinno Y *et al.* 1994	term	subset		x		P
Putatively placental imprinted genes									
ATP10A	ATPase, class V, type 10A	15q11.2	Steinhoff *et al.* 2009	term	na			x	M
CD81	CD81 molecule	11p15.5	McMinn *et al.* 2006	2nd/3rd-trimester	na			x	not imprinted
DCN	decorin	12q21.33	McMinn *et al.* 2006	2nd/3rd-trimester	na			x	unknown
DIO3	deiodinase, iodothyronine, type III	14q32	McMinn *et al.* 2006	2nd/3rd-trimester	na			x	unknown

Table I continued

Gene	Name	Location	References	Placental tissue	Mono-allelic expression	Parental expression in placenta			Parental expression (not placental-specific) [1]
						Paternal	Maternal	Not analysed	
GATM	glycine amidinotransferase (L-arginine:glycine amidinotransferase)	15q21.1	McMinn *et al.* 2006	2nd/3rd-trimester	na			x	unknown
GNAS	GNAS complex locus	20q13.3	Steinhoff *et al.* 2009, McMinn *et al.* 2006	term	na			x	isoform dependent
IGF2R	insulin-like growth factor 2 receptor	6q26	McMinn *et al.* 2006	2nd/3rd-trimester	na			x	not imprinted
			Monk *et al.* 2006	term	minor subset		x		not imprinted
KCNQ1	potassium voltage-gated channel, KQT-like subfamily, member 1	11p15.5	Steinhoff *et al.* 2009	term	na			x	M
MKRN1	makorin ring finger protein 1	7q34	McMinn *et al.* 2006	2nd/3rd-trimester	na			x	no record
PPP1CC	protein phosphatase 1, catalytic subunit, gamma isozyme	12q24.1-q24.2	McMinn *et al.* 2006	2nd/3rd-trimester	na			x	no record
ZIM2	zinc finger, imprinted 2	19q13.4	Steinhoff et al. 2009, McMinn *et al.* 2006	term	na			x	P

[1] Searched in imprinted gene catalogues (www.geneimprint.com, http://igc.otago.ac.nz). Na, not analysed; P, paternal; M, maternal.

McMinn *et al.* (2006) analysed whether altered expression of imprinted genes is implicated in non-syndromic human IUGR. They conducted a genome-wide survey of mRNA expression in late-gestation placental samples of 14 IUGR placentas compared to 15 non-IUGR placentas. Several imprinted genes were differentially expressed. They found for instance increased expression of the paternally imprinted *PHLDA2* and decreased expression of the maternally imprinted *MEST* and *PLAGL1* and paternally imprinted *MEG3, GATM* and *GNAS* in IUGR placentas (McMinn *et al.*, 2006). These results support the hypothesis that placental-specific imprinted genes are deregulated in IUGR.

A recent study systematically investigated the expression levels of 74 'putatively' imprinted genes in human late-gestation placental samples from 10 normal and 7 IUGR pregnancies by using quantitative RT-PCR (qRT-PCR) (Diplas *et al.*, 2009). They found that 52 of the 74 genes were expressed in human placental tissue of which 9 genes were significantly differentially expressed between both groups. Five genes were up-regulated (*PHLDA2, ILK2, NNAT, CCDC86*, *PEG10*) and four were down-regulated (*PLAGL1*, *DHCR24, ZNF331*, *CDKAL1*) in IUGR placentas, without a consistent 'parent-of-origin' pattern. They investigated LOI and found that 5 of these deregulated genes were not imprinted in placenta. Assessment of 14 placental imprinted genes revealed no correlation between expression and LOI, in both normal and IUGR placentas (Diplas *et al.*, 2009). Therefore, mechanisms other than LOI may be associated with the deregulated expression in IUGR placentas. Gene expression levels may vary according to the site of sampling. Even in uncomplicated pregnancies, intra-placenta variability has been described (Pidoux *et al.*, 2004; Wyatt *et al.*, 2005). This potential bias was also studied by Diplas *et al.* revealing no significant difference with respect to sampling variations (Diplas *et al.*, 2009).

Guo *et al.* investigated placentas of 20 control cases and 24 SGA with or without IUGR cases. They postulated that alterations in epigenetic control mechanisms leading to deregulation of growth-related imprinted genes on chromosome 11p15 may affect placental and fetal growth and lead to SGA/IUGR. They found that two imprinting clusters (KvDMR and *H19* DMR) maintained normal differential methylation in most SGA placentas, except for one, which showed loss of methylation at *H19* DMR. In this placenta, also LOI was demonstrated at the *H19* DMR as shown by bi-allelic expression of the *H19* gene. Furthermore, a significant decrease of *IGF2* expression, mostly independent of *H19* regulation, was found in all SGA placentas (Guo *et al.*, 2008).

Other researchers investigated the relation between placental imprinting and birthweight. The imprinted genes *IGF2, MEST, PHLDA2* and *IGF2R*, all with known roles in fetal growth, were studied in 200 normal term placentas (Apostolidou *et al.*, 2007). Elevated placental expression of *PHLDA2* was associated with lower birthweight. It is, however, not clear whether birthweight in this study was within normal ranges or whether also LBW or very LBW cases were included. Furthermore, these increased maternally expressed *PHLDA2* levels were independent of LOI (Apostolidou *et al.*, 2007). Indeed, several studies found that placental LOI usually seems to be unrelated to gene expression levels (Table II).

Epigenetics is also believed to be involved in the pathogenesis of pre-eclampsia. Several miRNAs are expressed in the human placenta (Barad *et al.*, 2004). Differential expression of specific placental miRNAs, among others miR-210, was found in pre-eclampsia placentas compared with normal placentas (Pineles *et al.*, 2007; Zhu *et al.*, 2009). Chelbi *et al.* suggest that an abnormal methylation pattern may be a common mechanism leading to pre-eclampsia (Chelbi *et al.*, 2008). Moreover, the proximal promoter of the non-imprinted *SERPINA3* is significantly hypomethylated at specific CpG positions in pre-eclampsia placentas compared with normal placentas (Chelbi *et al.*, 2007). Lastly, up-regulation of the expression of 13 tumour suppressor and growth regulatory genes has been identified in placentas of patients with severe early onset pre-eclampsia (Heikkila *et al.*, 2005).

There might also be a role for imprinted genes in the pathogenesis of pre-eclampsia. LOI of the imprinted *Cdkn1c* gene in a mouse model resulted in some of the features of pre-eclampsia, including hypertension and proteinuria (Kanayama *et al.*, 2002). Development of pre-eclampsia is associated with decreased invasive capacity of trophoblasts. Since down-regulation of *H19* expression is associated with development of choriocarcinoma and its highly invasive capacity (Walsh *et al.*, 1995; Kim *et al.*, 2003), Yu *et al*. investigated the hypothesis that alterations in paternal *H19* imprinting contribute to the pathogenesis of pre-eclampsia (Yu *et al.*, 2009). In the placentas of patients with pre-eclampsia associated with severe hypertension, indeed LOI of the *H19* gene was found (Yu *et al*Researchers have examined the role of aberrant DNA methylation in GTD. Trophoblast hyperplasia is a common feature of complete hydatidiform moles with the potential for malignant transformation to choriocarcinoma (Li *et al.*, 2002). Investigation of invasive choriocarcinoma

Table II Placental epigenetics in relation to placental-related pathologies.[1]

Pathology	Gene	Expressed allele	Methylation (investigated region)		Gene expression (fold change)	Mono-allelic expression (affected number)	Reference[2,3]
IUGR	CDKN1C	M	Na		↑ (3.3)	Na	McMinn *et al.* 2006
			Na		=	Na	Diplas *et al.* 2009
	DLK1	P	Na		=	LOI (2/5)	Diplas *et al.* 2009
	GATM	M	Na		↓ (0.59)	Na	McMinn *et al.* 2006
			Na		Na	Na	Diplas *et al.* 2009
	GNAS	M	Na		↓ (?)	Na	McMinn *et al.* 2006
			Na		Na	Na	Diplas *et al.* 2009
	H19	M	Na		=	LOI (1/5)	Diplas *et al.* 2009
	IGF2	P	Na		↓ (0.39)	Na	McMinn *et al.* 2006
			Na		=	=[4]	Diplas *et al.* 2009
	MEG3	M	Na		↓ (0.52)	Na	McMinn *et al.* 2006
			Na		=	=	Diplas *et al.* 2009
	MEST	P	=		↓ (0.72)	Na	McMinn *et al.* 2006
			Na		=	=[4]	Diplas *et al.* 2009
	NNAT	P	Na		=	Na	McMinn *et al.* 2006
			Na		↑	Na	Diplas *et al.* 2009
	PEG10	P	Na		=	Na	McMinn *et al.* 2006
			Na		↑	=	Diplas *et al.* 2009
	PHLDA2	M	=	(KvDMR)	↑ (1.27)	Na	McMinn *et al.* 2006
			Na		↑	=	Diplas *et al.* 2009
	PLAGL1	P	Na		↓ (0.62)	Na	McMinn *et al.* 2006
			Na		↓	LOI (2/13)	Diplas *et al.* 2009
	SERPINA3	M/P	=		↑		Chelbi *et al.* 2007
	SERPINB2	M/P	Na		↓		Chelbi *et al.* 2007

Table II continued

Pathology	Gene	Expressed allele	Methylation (investigated region)		Gene expression (fold change)	Mono-allelic expression (affected number)	Reference[2,3]
	SERPINB7	M/P	Na		↑		Chelbi *et al.* 2007
	SNRPN	P	Na		=	LOI (2/8)	Diplas *et al.* 2009
SGA	CDKN1C	M	=	(KvDMR)	Na	=	Guo *et al.* 2008
	H19	M	LOM (1/24))	(H19DMR,	=	LOI (1/24)	Guo *et al.* 2008
	IGF2	P	LOM (1/24))	(H19DMR,	↓ (0.57)	=	Guo *et al.* 2008
	KCNQ1OT1	P	=	(KvDMR)	Na	=	Guo *et al.* 2008
	PHLDA2	M	=	(KvDMR)	Na	=	Guo *et al.* 2008
Lower birthweight	IGF2	M	Na		=	=	Apostolidou *et al.* 2007
	MEST	P	Na		=	=[5]	Apostolidou *et al.* 2007
	PHLDA2	M	Na		↑	=	Apostolidou *et al.* 2007
Pre-eclampsia	H19	M	Na		=	LOI (6/13)	Yu *et al.* 2009
	SERPINA1	M/P	Na		↓	Nr	Chelbi *et al.* 2007
	SERPINA3	M/P	LOM		=	Nr	Chelbi *et al.* 2007
	SERPINB2	M/P	Na		↓	Nr	Chelbi *et al.* 2007
	miR-210	M/P	Na		↑ (3.0)	Na	Pineles *et al.* 2007
			Na		↑ (3.64)	Na	Zhu *et al.* 2009
	miR-182	M/P	Na		↑ (2.1)	Na	Pineles *et al.* 2007
GTD	OCT4	M/P	GOM		↓	Nr	Zhang *et al.* 2008
	p16	M/P	GOM		↓	Nr	Xue *et al.* 2004
	HIC-1	M/P	GOM		↓	Nr	Xue *et al.* 2004
	TIMP3	M/P	GOM		Na	Nr	Xue *et al.* 2004
	GSTP1	M/P	=		Na	Nr	Xue *et al.* 2004
	DSAPK	M/P	=		Na	Nr	Xue *et al.* 2004
	E-cadherin	M/P	GOM		↓	Nr	Xue *et al.* 2004

[1] The effects mentioned in the table are those from the affected group compared with a control group. In almost all studies third trimester/term placentas are investigated. Only Xue *et al.* 2004 and Zhang *et al.* 2008 investigated, respectively, first trimester and first trimester and term placentas.
[2] McMinn *et al.* 2006 describe a microarray study and Diplas *et al.* 2009 analysed 74 imprinted genes. For both studies only the imprinted genes that were differentially expressed in one of the two studies are described.
[3] Zhu *et al.* 2009 describe a microarray analysis of miRNAs. Thirty-four miRNAs are deregulated, but only the miRNA that was found to be affected in the study of Pineles *et al.* 2007 is reported.
[4] LOI was reported in some IUGR placentas, but this was comparable with the LOI reported in the non-IUGR placentas
[5] LOI was reported in 8 of 42 placentas, but this was not correlated to birthweight.
GOM, gain of methylation; GTD, gestational trophoblastic disease; IUGR, intrauterine growth reduction; LOI, loss of imprinting; LOM, loss of methylation; Na, not analysed; Nr, not relevant; SGA, small for gestational age; '=', not affected as compared with control.

cell lines displayed altered methylation patterns consistent with a role of methylation change in GTD (Novakovic *et al.*, 2008). The transcription factor *Oct4,* plays a crucial role in maintaining pluripotency of embryonic stem cells and its hypermethylation is associated with the differentiation of TE cell lineage, from which the normal placenta derives. Both methylated and unmethylated *Oct4* alleles were observed in normal placenta, while 33% of hydatidiform moles and two choriocarcinoma cell lines displayed only methylated alleles. Down-regulation of *Oct 4* by hypermethylation implies an important role in the pathogenesis of GTD (Zhang *et al.*, 2008). In addition, aberrant tumour suppressor gene methylation status has been associated with GTD, specifically hydatidiform mole and choriocarcinoma (Xue *et al.*, 2004).

Environmental effects

Increasing evidence suggests that the environment during pre- and post-natal development can affect the risk on chronic diseases such as cancer, cardiovascular disease, diabetes, obesity and behavioural disorders like schizophrenia by altering epigenetic programming (Jirtle *et al.*, 2007). A well-known example is the effect of maternal diet on coat colour and obesity in A^{vy}/a mice offspring (Waterland *et al.*, 2003). Also other environmental factors like tobacco smoke, alcohol, radiation and chemicals have been shown to influence disease susceptibility and can even induce transgenerational phenotypic effects (Singh *et al.*, 2003; Feil, 2006; Jirtle *et al.*, 2007; Baccarelli *et al.*, 2009). One of the best known examples in humans is the effect of diethylstilbestrol (DES), which was given to pregnant women in the sixties, to prevent miscarriages (Li *et al.*, 2003). This chemical, however, leads to uterus malformations in the offspring, caused by epigenetic deregulation (Li *et al.*, 2003; Bromer *et al.*, 2009; Sato *et al.*, 2009).

Table III Environmental effects on placental epigenetics.[1]

Condition	Time of exposure	Gene	Species	Expressed allele	Methylation		Gene expression	Mono-allelic expression	Reference[4,5]
Ethanol	At 1.5 and 2.5dpc	H19	mouse	M	LOM	(H19DMR)	Na	Na	Haycock *et al.* 2009
Ovulation induction and *in vivo* development or embryo transfer	At 3.5dpc	H19	mouse	M	=	(H19DMR)	Na	LOI (22/36)	Fortier *et al.* 2008
		Igf2	mouse	P	Na		↑ (3.4)	=	Fortier *et al.* 2008
		Kcnq1ot1	mouse	P	Na		Na	=	Fortier *et al.* 2008
		Snrpn	mouse	P	=	(SnrpnDMR)	Na	LOI (10/36)	Fortier *et al.* 2008
Embryo transfer (with embryo culture)	2-cell embryo collection + culture till blastocyst stage	Ascl2	mouse	M	LOM	(KvDMR)	↑ (12.5)	Na	Rivera *et al.* 2008
		Cdkn1c	mouse	M	LOM	(KvDMR1)	Na	LOI (1/28)	Rivera *et al.* 2008
		H19	mouse	M	LOM	(H19DMR)	=	LOI (19/28)	Rivera *et al.* 2008
		Igf2	mouse	P	Na		↓ (0.4)	=	Rivera *et al.* 2008
		Kcnq1	mouse	M	LOM	(KvDMR1)	Na	LOI (6/27)	Rivera *et al.* 2008
		Kcnq1ot1	mouse	P	LOM	(KvDMR1)	Na	LOI (9/27)	Rivera *et al.* 2008
		Mkrn3	mouse	P	Na		Na	LOI (26/28)[2]	Rivera *et al.* 2008
		Peg3	mouse	P	Na		Na	LOI (8/28)	Rivera *et al.* 2008
		Snrpn	mouse	P	Na		Na	LOI (8/27)	Rivera *et al.* 2008
		Zim1	mouse	M	Na		Na	=	Rivera *et al.* 2008
Culture	2-cell embryo collection + culture till blastocyst stage	Ascl2	mouse	M	Na		Na	LOI	Mann *et al.* 2004
		H19	mouse	M	LOM	(H19DMR)	Na	LOI	Mann *et al.* 2004
		Peg3	mouse	P	Na		Na	LOI	Mann *et al.* 2004
		Snrpn	mouse	P	LOM	(SnrpnDMR)	Na	LOI	Mann *et al.* 2004
		Xist	mouse	In females M or P	Na		Na	LOI[3]	Mann *et al.* 2004
ART		COPG2	human	P	=		↓	Na	Katari *et al.* 2009
		DLK1	human	P	=		Na	Na	Katari *et al.* 2009
					=		Na	Na	Tierling *et al.* 2010
		GNAS	human	M	=		=	Na	Katari *et al.* 2009

Table III continued

Condition	Time of exposure	Gene	Species	Expressed allele	Methylation		Gene expression	Mono-allelic expression	Reference[4,5]
					=	(4 DMRs)	Na	Na	Tierling *et al.* 2010
		GRB10	human	P	LOM		Na	Na	Katari *et al.* 2009
					=		Na	Na	Tierling *et al.* 2010
		H19	human	M	=		Na	Na	Katari *et al.* 2009
					=		Na	Na	Tierling *et al.* 2010
		KCNQ1OT1	human	P	=		Na	Na	Katari *et al.* 2009
					=	(KvDMR1)	Na	Na	Tierling *et al.* 2010
		MEST	human	P	LOM GOM	(2CpGs) (1CpG)	↑ (2.09)	Na	Katari *et al.* 2009
					=		Na	Na	Tierling *et al.* 2010
		NNAT	human	P	=		↓ (0.55)	Na	Katari *et al.* 2009
		PEG3	human	P	LOM GOM	(3CpGs) (2CpGs)	=	Na	Katari *et al.* 2009
		SERPINF1	human	M/P	LOM		↑ (1.81)	Nr	Katari *et al.* 2009
		SLC22A2	human	M	LOM		=	Na	Katari *et al.* 2009
		SNRPN	human	P	=		Na	Na	Katari *et al.* 2009
					=		Na	Na	Tierling *et al.* 2010
Hypoxia	*In vitro* exposure of term trophoblast	miR-93	human	M/P	Na		↑	Na	Donker *et al.*2007
		miR-424	human	M/P	Na		↓	Na	Donker *et al.*2007

[1] The effects mentioned in the table are those from the exposed group compared with a control group.
[2] Mkrn3 displayed also severe LOI in control samples, but the LOI was more severe in placentas from cultured and transferred embryos.
[3] Xist was inappropriately expressed in male placental tissue after embryo culture.
[4] Katari *et al.* 2009 performed an array-based methylation analysis. Forty genes were different between the *in vivo* and *in vitro* group. Of these only the ones subject to further expression analysis or the ones affected according to the study by Tierling *et al.* 2010 are reported here.
[5] In the study by Tierling *et al.* 2010 chorion and amnion samples are analysed, instead of throphoblast as with the other studies.
CD, conflicting data; GOM, gain of methylation; ID, isoform dependant; IUGR, intrauterine growth reduction; LOI, loss of imprinting; LOM, loss of methylation; Na, not analysed; Nr, not relevant; '=', not affected when compared with control.

There are several studies which investigated the relationship between environmental effects and placental epigenetics (Table III). Haycock *et al.* used a mouse model to investigate whether disruption of imprinting control at the *H19* ICR may be a mechanism of ethanol-induced growth retardation in the event of ethanol exposure during fetal development (Haycock *et al.*, 2009). Ethanol exposed midgestation placentas and embryos were severely growth retarded when compared to controls. DNA methylation was unaffected in embryos, although the paternal alleles were significantly less methylated in ethanol exposed placentas. Data showing a relationship between placental weight and ethanol treatment suggested that this was partially dependent on DNA methylation at the CCCTC-binding factor (CTCF) site on the paternal allele in placentas (Haycock *et al.*, 2009). Unfortunately, gene expression was not analysed.

Unlike most cells, placental cytotrophoblasts proliferate in response to hypoxia (Adelman *et al.*, 1999). An important regulator of the responses of a cell to oxygen tension is the hypoxia-inducible factor 1 (HIF1) (Maltepe *et al.*, 2005). For normal trophoblast differentiation, crosstalk between the HIFs and HDACs is crucial, which links HIF function with epigenetic regulatory mechanisms (Maltepe *et al.*, 2005). Donker et al. (2007) investigated *in vitro* the effect of varying oxygen levels on miRNAs in term human trophoblasts during differentiation and in hypoxic environment. In hypoxic trophoblasts, the expression of miR-93 was up-regulated, whereas the expression of miR-424 was down-regulated. These results indicate that the miRNA biosynthetic pathway is functional even in a hypoxic environment, and that hypoxia regulates the expression level of miRNAs in these cells (Donker *et al.*, 2007).

Assisted reproduction technologies (ART) are increasingly used worldwide in animals and humans to achieve (higher) pregnancy and life-birth rates in subfertile patients. ART in humans have been associated with LBWs, SGA, preterm deliveries (Helmerhorst *et al.*, 2004; Jackson *et al.*, 2004; McGovern *et al.*, 2004) and birth defects (Rimm *et al.*, 2004; Hansen *et al.*, 2005; Lie *et al.*, 2005). Furthermore, several groups have raised concerns about a possible association of rare imprinting diseases with ART. Analysis of affected ART children have shown methylation defects at the DMRs of *SNRPN* (Angelman Syndrome), *KCNQ1OT1* (Beckwith-Wiedemann Syndrome) and *PEG1/MEST* (Silver-Russell Syndrome) (Cox *et al.*, 2002; DeBaun *et al.*, 2003; Gicquel *et al.*, 2003; Maher *et al.*, 2003; Halliday *et al.*, 2004; Kagami *et al.*, 2007). In women undergoing ART, the risk of

pre-eclampsia, stillbirth and placenta praevia is significantly increased (Jackson *et al.*, 2004). Several aspects of ART, such as ovulation induction and embryo culture, have been investigated as a possible cause of imprinting disturbance.

Induction of ovulation with high doses of gonadotrophin is used to obtain multiple mature oocytes. This treatment may force oocytes to undergo the growth and maturation phases too rapidly or rescue oocytes already selected for atresia, possibly leading to inadequate establishment or maintenance of the parent-of-origin effect (Fortier *et al.*, 2008). The timing of imprint establishment in human adult oogenesis is mostly unknown, but in mice it starts post-natally, during the oocyte growth phase, in a locus-specific manner with some genes achieving their full methylation status only very late in oocyte growth (Bao *et al.*, 2000; Obata *et al.*, 2002; Lucifero *et al.*, 2004b). According to earlier mouse studies, ovulation induction results in delayed embryo development, decreased implantation rates and increased post-implantation loss (Fossum *et al.*, 1989; Ertzeid *et al.*, 1992, 2001; Van der Auwera *et al.*, 2001). Also, the effect of ovulation induction on DNA methylation and imprinting was investigated. One study in both mouse and human oocytes showed no difference in imprint establishment following ovulation induction in the maternally imprinted DMRs of *MEST, KCNQ1OT1* and *ZAC/PLAGL1* (Sato *et al.*, 2007). Surprisingly, increased DNA methylation was found at the paternally imprinted *H19* DMR in superovulated oocytes, which is normally unmethylated in oocytes (Sato *et al.*, 2007). Ovulation induction also induces aberrant expression of the imprinted H19 gene in mouse blastocysts (Fauque *et al.*, 2007) with a normal methylation pattern at its regulatory sequence. Contradictory, in individual blastocyst stage mouse embryos, ovulation induction perturbed methylation in both maternally and paternally imprinted DMRs (loss of *Snrpn, Peg3, Kcnq1ot1;* gain of *H19* methylation) (Market-Velker *et al.*, 2010). Aberrant imprinted methylation was also dose-dependent, being more frequent at higher hormone dosages (Market-Velker *et al.*, 2010). These studies altogether suggest that ovulation induction might interfere with imprint establishment as well as with imprint maintenance after fertilization. Whether the possible effect of ovulation induction is still present in placental tissue is less well investigated. Indeed, one study reported bi-allelic expression of *H19* and *Snrpn,* and bi-allelic expression of *H19* in 9.5dpc placentas after ovulation induction/*in vivo* development and ovulation induction/3.5dpc embryo transfer, respectively (Fortier *et al.*, 2008). There was normal mono-allelic expression of these genes

in the embryo. These results suggest an apparently primary effect of ovulation induction on the expression of imprinted genes in the placenta when compared with the embryo. The expression of *Igf2*, an important placental growth factor, was increased following ovulation induction despite retaining mono-allelic expression (Fortier *et al.*, 2008). Earlier in this review, we discussed that the placenta displays lower overall methylation levels compared with the embryo. Fortier *et al.* also noted that the overall levels of methylation at the DMRs of both *Snrpn* and *H19* were lower in placentas than in embryos, without an effect on the mono-allelic expression of these genes (Fortier *et al.*, 2008). This could suggest again that imprint maintenance in the placenta is regulated primarily by histone modifications instead of by DNA methylation.

The role of oxygen tension during embryo culture was studied in both animal and human studies. In preimplantation mouse embryos, culture in 20% oxygen resulted in far greater perturbations in the global pattern of gene regulation than culture in 5% oxygen, when compared with embryos that developed *in vivo* (Rinaudo *et al.*, 2006). In humans, such perturbations might be the cause of the less optimal embryo development (Kovacic *et al.*, 2008; Waldenstrom *et al.*, 2009) and lower rates of live birth implantation and live births (Meintjes *et al.*, 2009; Waldenstrom *et al.*, 2009) after culture in an atmospheric (19-21%) oxygen environment. Preimplantation culture of embryos can also influence birthweight in a negative and positive manner through changes in culture condition. Recently, research in humans has indicated that the type of medium used for culturing IVF embryos during the first few days after fertilization significantly affects the birthweight of the resulting human newborns (Dumoulin *et al.*, 2010). Overgrowth abnormalities, collectively referred to as 'large offspring syndrome', are frequently seen in sheep and cattle (Thompson *et al.*, 1995; Sinclair *et al.*, 2000). Preimplantation embryo culture has been shown to affect methylation and expression of imprinted genes in several animal models. In sheep, *in vitro* culture with serum until the blastocyst stage, gives rise to fetuses that show aberrant methylation and lack of expression of a well-known imprinted locus *Igf2r* leading to large offspring (Young *et al.*, 2001). The addition of fetal calf serum to M16 medium for mouse embryonic culture, also alters the expression of imprinted genes but contrarily reduces the birthweight (Khosla *et al.*, 2001). Not all imprinted genes were affected (Doherty *et al.*, 2000; Khosla *et al.*, 2001), but especially H19 seems to be vulnerable for culture medium-induced aberrations of methylation and expression (Sasaki *et*

al., 1995; Doherty *et al.*, 2000; Mann *et al.*, 2004). Only a subset of individual cultured mouse blastocysts were affected (Mann *et al.*, 2004). Moreover, proper imprinted expression for the most part was preserved in the embryo, while placental tissues displayed activation of the normally silent allele for *H19, Ascl2, Snrpn, Peg3* and *Xist* (Mann *et al.*, 2004). Embryo transfer, with or without embryo culture, also led to LOI in placental tissue but not in the embryo (Rivera *et al.*, 2008). This indicates that appropriate imprinting is not restored during post-implantation development of the placenta. The findings of Mann *et al.* and Rivera et al. parallel the findings of Haycock *et al.* (ethanol) and Fortier *et al. (*ovulation induction) suggesting that tissues of TE origin are more sensitive to preimplantation epigenetic disturbance than embryonic tissues.

Very recently, the complete placental transcriptome after ART in mice was analysed, revealing a substantial alteration of placental gene expression at 10.5 days post-fertilization (Fauque *et al.*, 2010). About 6% of placental transcripts were altered at the 2-fold threshold, with most genes being down-regulated, after IVF and embryo culture compared with placentas of embryos produced *in vivo*. Conversely, imprinted genes, as well as X-linked genes, were overall more up-regulated than the rest of the transcriptome (Fauque *et al.*, 2010). Interestingly, analysis of some imprinted genes showed that paternally expressed genes were primarily up-regulated. Moreover, genes involved in cellular proliferation and cell cycle pathways were induced, whereas genes involved in angiogenic signalling and the immune system were reduced (Fauque *et al.*, 2010). These findings, although in mice, may explain future placental problems, e.g. pre-eclampsia, as seen after ART in humans (Jackson *et al.*, 2004).

So far, few studies have been conducted to examine the effect of ART on human placental epigenetics. Recently, Gomes *et al.* reported a higher incidence of hypomethylation of the KvDMR1 in clinically normal children conceived by ART. They investigated blood samples and found aberrant hypomethylation in 3 ART children (3/18) and in none of the 30 non-ART newborns (Gomes *et al.*, 2009). Interestingly, each of these three children had a dizygotic sibling with discordant methylation. Also placental tissue was obtained. Unfortunately, no placental samples were collected after birth of the hypomethylated children. Katari *et al.* performed an array-based methylation analysis in placenta and cord blood of 10 ART and 13 non-ART children (Katari *et al.*, 2009). Moderate but significant methylation differences were observed between groups with the

ART children displaying lower mean methylation levels at specific CpG sites in placenta and higher mean methylation levels in cord blood (Katari *et al.*, 2009). In contrast, Tierling *et al.* found no significant methylation difference at 10 DMRs in amnion/chorion tissue and umbilical cord blood of 185 phenotypically normal children, comprising 77 children conceived by ICSI, 35 by IVF and 73 spontaneously (Tierling *et al.*, 2010). However, the latter studies both found that MEST was hypomethylated in placental cells compared with umbilical cord blood.

Conclusion and future prospects

In recent years, there has been an explosion of knowledge in the field of epigenetics. Epigenetics is gradually recognized as having an important role in placental development and functioning. Proper epigenetic regulation of imprinted genes in particular, but also of non-imprinted genes is crucial in the placenta. This epigenetic regulation evolves during preimplantation development and further gestation. Environmental effects can disturb placental epigenetics and herewith placental development and function, with possible downstream consequences for maternal morbidity, fetal development and disease susceptibility in later life (summarized in Figure I). Especially since several animal studies have suggested that placental tissues are more sensitive to preimplantation epigenetic disturbance than embryonic tissues and ART are increasingly used worldwide, this emphasizes the need for further investigations in human placentas. Placental tissue sampled after placental-related pathologies and from pregnancies resulting from ART, can be used to screen for epigenetic disturbances, instead of being discarded as a meaningless by-product. Early screening using this tissue may lead to earlier intervention or better surveillance of children who may be at risk later in life. With epigenetic marks being reversible, epigenetic therapy may eventually provide promising new challenges. This also implies the need for more fundamental research to investigate the involvement of histone modifications and ncRNAs in placental gene regulation.

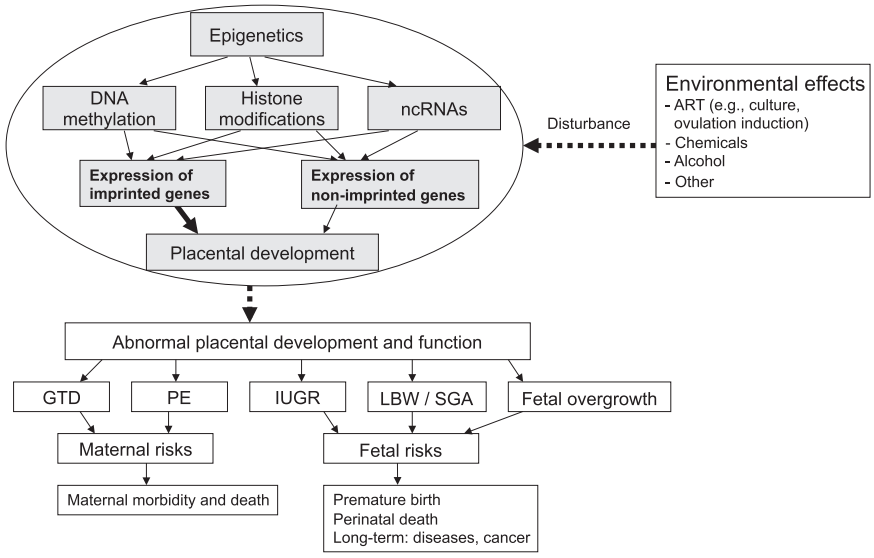


Figure I The role of epigenetics in placental development and the possible consequences of its disturbance which can be caused by environmental effects.

Arrows indicate relationships between the different steps. Bold arrow indicates the importance of imprinted genes for placental development. Dashed arrows indicate the effect of environment on placental disturbance. ncRNAs, non-coding RNAs; ART, assisted reproduction technologies; GTD, gestational trophoblastic disease; PE, pre-eclampsia; IUGR, intrauterine growth restriction; LBW, low birthweight; SGA, small for gestational age.

Acknowledgements

We thank Professor J.P.M. Geraedts for his insightful comment on our manuscript.

References

Adelman DM, Maltepe E,Simon MC. Multilineage embryonic hematopoiesis requires hypoxic ARNT activity. *Genes Dev* 1999;13:(19): 2478-2483.

Alsat E, Guibourdenche J, Couturier A,Evain-Brion D. Physiological role of human placental growth hormone. *Mol Cell Endocrinol* 1998;140:(1-2): 121-127.

Anderson MG, Nakane M, Ruan X, Kroeger PE,Wu-Wong JR. Expression of VDR and CYP24A1 mRNA in human tumors. *Cancer Chemother Pharmacol* 2006;57:(2): 234-240.

Angiolini E, Fowden A, Coan P, Sandovici I, Smith P, Dean W, Burton G, Tycko B, Reik W, Sibley C*, et al.* Regulation of placental efficiency for nutrient transport by imprinted genes. *Placenta* 2006;27 Suppl A: S98-102.

Apostolidou S, Abu-Amero S, O'Donoghue K, Frost J, Olafsdottir O, Chavele KM, Whittaker JC, Loughna P, Stanier P,Moore GE. Elevated placental expression of the imprinted PHLDA2 gene is associated with low birth weight. *J Mol Med* 2007;85:(4): 379-387.

Arima T, Hata K, Tanaka S, Kusumi M, Li E, Kato K, Shiota K, Sasaki H,Wake N. Loss of the maternal imprint in Dnmt3Lmat-/- mice leads to a differentiation defect in the extraembryonic tissue. *Dev Biol* 2006;297:(2): 361-373.

Baccarelli A,Bollati V. Epigenetics and environmental chemicals. *Curr Opin Pediatr* 2009;21:(2): 243-251.

Bamberg C,Kalache KD. Prenatal diagnosis of fetal growth restriction. *Semin Fetal Neonatal Med* 2004;9:(5): 387-394.

Bao S, Obata Y, Carroll J, Domeki I,Kono T. Epigenetic modifications necessary for normal development are established during oocyte growth in mice. *Biol Reprod* 2000;62:(3): 616-621.

Barad O, Meiri E, Avniel A, Aharonov R, Barzilai A, Bentwich I, Einav U, Gilad S, Hurban P, Karov Y*, et al.* MicroRNA expression detected by oligonucleotide microarrays: system establishment and expression profiling in human tissues. *Genome Res* 2004;14:(12): 2486-2494.

Barker DJ, Hales CN, Fall CH, Osmond C, Phipps K,Clark PM. Type 2 (non-insulin-dependent) diabetes mellitus, hypertension and hyperlipidaemia (syndrome X): relation to reduced fetal growth. *Diabetologia* 1993;36:(1): 62-67.

Barlow DP. Methylation and imprinting: from host defense to gene regulation? *Science* 1993;260:(5106): 309-310.

Barton SC, Surani MA,Norris ML. Role of paternal and maternal genomes in mouse development. *Nature* 1984;311:(5984): 374-376.

Beaujean N, Hartshorne G, Cavilla J, Taylor J, Gardner J, Wilmut I, Meehan R,Young L. Non-conservation of mammalian preimplantation methylation dynamics. *Curr Biol* 2004;14:(7): R266-267.

Bird A. DNA methylation patterns and epigenetic memory. *Genes Dev* 2002;16:(1): 6-21.

Bird A. Perceptions of epigenetics. *Nature* 2007;447:(7143): 396-398.

Bodnar LM, Catov JM, Simhan HN, Holick MF, Powers RW,Roberts JM. Maternal vitamin D deficiency increases the risk of preeclampsia. *J Clin Endocrinol Metab* 2007;92:(9): 3517-3522.

Bourc'his D,Bestor TH. Meiotic catastrophe and retrotransposon reactivation in male germ cells lacking Dnmt3L. *Nature* 2004;431:(7004): 96-99.

Bourc'his D, Xu GL, Lin CS, Bollman B,Bestor TH. Dnmt3L and the establishment of maternal genomic imprints. *Science* 2001;294:(5551): 2536-2539.

Boyer LA, Plath K, Zeitlinger J, Brambrink T, Medeiros LA, Lee TI, Levine SS, Wernig M, Tajonar A, Ray MK*, et al.* Polycomb complexes repress developmental regulators in murine embryonic stem cells. *Nature* 2006;441:(7091): 349-353.

Boyko EJ. Proportion of type 2 diabetes cases resulting from impaired fetal growth. *Diabetes Care* 2000;23:(9): 1260-1264.

Bromer JG, Wu J, Zhou Y,Taylor HS. Hypermethylation of homeobox A10 by in utero diethylstilbestrol exposure: an epigenetic mechanism for altered developmental programming. *Endocrinology* 2009;150:(7): 3376-3382.

Buiting K, Saitoh S, Gross S, Dittrich B, Schwartz S, Nicholls RD,Horsthemke B. Inherited microdeletions in the Angelman and Prader-Willi syndromes define an imprinting centre on human chromosome 15. *Nat Genet* 1995;9:(4): 395-400.

Carninci P, Kasukawa T, Katayama S, Gough J, Frith MC, Maeda N, Oyama R, Ravasi T, Lenhard B, Wells C, *et al.* The transcriptional landscape of the mammalian genome. *Science* 2005;309:(5740): 1559-1563.

Chaddha V, Viero S, Huppertz B,Kingdom J. Developmental biology of the placenta and the origins of placental insufficiency. *Semin Fetal Neonatal Med* 2004;9:(5): 357-369.

Chapman V, Forrester L, Sanford J, Hastie N,Rossant J. Cell lineage-specific undermethylation of mouse repetitive DNA. *Nature* 1984;307:(5948): 284-286.

Charalambous M, da Rocha ST,Ferguson-Smith AC. Genomic imprinting, growth control and the allocation of nutritional resources: consequences for postnatal life. *Curr Opin Endocrinol Diabetes Obes* 2007;14:(1): 3-12.

Chelbi ST, Mondon F, Jammes H, Buffat C, Mignot TM, Tost J, Busato F, Gut I, Rebourcet R, Laissue P, *et al.* Expressional and epigenetic alterations of placental serine protease inhibitors: SERPINA3 is a potential marker of preeclampsia. *Hypertension* 2007;49:(1): 76-83.

Chelbi ST,Vaiman D. Genetic and epigenetic factors contribute to the onset of preeclampsia. *Mol Cell Endocrinol* 2008;282:(1-2): 120-129.

Chiu RW, Chim SS, Wong IH, Wong CS, Lee WS, To KF, Tong JH, Yuen RK, Shum AS, Chan JK, *et al.* Hypermethylation of RASSF1A in human and rhesus placentas. *Am J Pathol* 2007;170:(3): 941-950.

Chuang HC, Chang CW, Chang GD, Yao TP,Chen H. Histone deacetylase 3 binds to and regulates the GCMa transcription factor. *Nucleic Acids Res* 2006;34:(5): 1459-1469.

Coan PM, Burton GJ,Ferguson-Smith AC. Imprinted genes in the placenta--a review. *Placenta* 2005;26 Suppl A: S10-20.

Coan PM, Fowden AL, Constancia M, Ferguson-Smith AC, Burton GJ,Sibley CP. Disproportional effects of Igf2 knockout on placental morphology and diffusional exchange characteristics in the mouse. *J Physiol* 2008;586:(Pt 20): 5023-5032.

Constancia M, Hemberger M, Hughes J, Dean W, Ferguson-Smith A, Fundele R, Stewart F, Kelsey G, Fowden A, Sibley C, *et al.* Placental-specific IGF-II is a major modulator of placental and fetal growth. *Nature* 2002;417:(6892): 945-948.

Cox GF, Burger J, Lip V, Mau UA, Sperling K, Wu BL,Horsthemke B. Intracytoplasmic sperm injection may increase the risk of imprinting defects. *Am J Hum Genet* 2002;71:(1): 162-164.

Cross JC. The genetics of pre-eclampsia: a feto-placental or maternal problem? *Clin Genet* 2003;64:(2): 96-103.

Curhan GC, Chertow GM, Willett WC, Spiegelman D, Colditz GA, Manson JE, Speizer FE,Stampfer MJ. Birth weight and adult hypertension and obesity in women. *Circulation* 1996a;94:(6): 1310-1315.

Curhan GC, Willett WC, Rimm EB, Spiegelman D, Ascherio AL,Stampfer MJ. Birth weight and adult hypertension, diabetes mellitus, and obesity in US men. *Circulation* 1996b;94:(12): 3246-3250.

DeBaun MR, Niemitz EL,Feinberg AP. Association of in vitro fertilization with Beckwith-Wiedemann syndrome and epigenetic alterations of LIT1 and H19. *Am J Hum Genet* 2003;72:(1): 156-160.

Delcuve GP, Rastegar M,Davie JR. Epigenetic control. *J Cell Physiol* 2009;219:(2): 243-250.

DeLuca HF. Overview of general physiologic features and functions of vitamin D. *Am J Clin Nutr* 2004;80:(6 Suppl): 1689S-1696S.

Devriendt K. Hydatidiform mole and triploidy: the role of genomic imprinting in placental development. *Hum Reprod Update* 2005;11:(2): 137-142.

Diplas AI, Lambertini L, Lee MJ, Sperling R, Lee YL, Wetmur J,Chen J. Differential expression of imprinted genes in normal and IUGR human placentas. *Epigenetics* 2009;4:(4): 235-240.

Doherty AS, Mann MR, Tremblay KD, Bartolomei MS,Schultz RM. Differential effects of culture on imprinted H19 expression in the preimplantation mouse embryo. *Biol Reprod* 2000;62:(6): 1526-1535.

Dokras A, Coffin J, Field L, Frakes A, Lee H, Madan A, Nelson T, Ryu GY, Yoon JG,Madan A. Epigenetic regulation of maspin expression in the human placenta. *Mol Hum Reprod* 2006;12:(10): 611-617.

Donker RB, Mouillet JF, Nelson DM,Sadovsky Y. The expression of Argonaute2 and related microRNA biogenesis proteins in normal and hypoxic trophoblasts. *Mol Hum Reprod* 2007;13:(4): 273-279.

Dumoulin JC, Land JA, Van Montfoort AP, Nelissen EC, Coonen E, Derhaag JG, Schreurs IL, Dunselman GA, Kester AD, Geraedts JP, *et al.* Effect of in vitro culture of human embryos on birthweight of newborns. *Hum Reprod* 2010;25:(3): 605-612.

Dusso AS, Brown AJ,Slatopolsky E. Vitamin D. *Am J Physiol Renal Physiol* 2005;289:(1): F8-28.

Edwards CA,Ferguson-Smith AC. Mechanisms regulating imprinted genes in clusters. *Curr Opin Cell Biol* 2007;19:(3): 281-289.

Engstrom PG, Suzuki H, Ninomiya N, Akalin A, Sessa L, Lavorgna G, Brozzi A, Luzi L, Tan SL, Yang L, *et al.* Complex Loci in human and mouse genomes. *PLoS Genet* 2006;2:(4): e47.

Eriksson JG, Forsen TJ, Osmond C,Barker DJ. Pathways of infant and childhood growth that lead to type 2 diabetes. *Diabetes Care* 2003;26:(11): 3006-3010.

Ertzeid G,Storeng R. Adverse effects of gonadotrophin treatment on pre- and postimplantation development in mice. *J Reprod Fertil* 1992;96:(2): 649-655.

Ertzeid G,Storeng R. The impact of ovarian stimulation on implantation and fetal development in mice. *Hum Reprod* 2001;16:(2): 221-225.

Esteve PO, Chin HG, Smallwood A, Feehery GR, Gangisetty O, Karpf AR, Carey MF,Pradhan S. Direct interaction between DNMT1 and G9a coordinates DNA and histone methylation during replication. *Genes Dev* 2006;20:(22): 3089-3103.

Fauque P, Jouannet P, Lesaffre C, Ripoche MA, Dandolo L, Vaiman D,Jammes H. Assisted Reproductive Technology affects developmental kinetics, H19 Imprinting Control Region methylation and H19 gene expression in individual mouse embryos. *BMC Dev Biol* 2007;7: 116.

Fauque P, Mondon F, Letourneur F, Ripoche MA, Journot L, Barbaux S, Dandolo L, Patrat C, Wolf JP, Jouannet P, *et al.* In vitro fertilization and embryo culture strongly impact the placental transcriptome in the mouse model. *PLoS One* 2010;5:(2): e9218.

Feil R. Environmental and nutritional effects on the epigenetic regulation of genes. *Mutat Res* 2006;600:(1-2): 46-57.

Ferguson-Smith AC, Moore T, Detmar J, Lewis A, Hemberger M, Jammes H, Kelsey G, Roberts CT, Jones H,Constancia M. Epigenetics and imprinting of the trophoblast -- a workshop report. *Placenta* 2006;27 Suppl A: S122-126.

Ferretti C, Bruni L, Dangles-Marie V, Pecking AP,Bellet D. Molecular circuits shared by placental and cancer cells, and their implications in the proliferative, invasive and migratory capacities of trophoblasts. *Hum Reprod Update* 2007;13:(2): 121-141.

Fisher SJ. The placental problem: linking abnormal cytotrophoblast differentiation to the maternal symptoms of preeclampsia. *Reprod Biol Endocrinol* 2004;2: 53.

Fortier AL, Lopes FL, Darricarrere N, Martel J,Trasler JM. Superovulation alters the expression of imprinted genes in the midgestation mouse placenta. *Hum Mol Genet* 2008;17:(11): 1653-1665.

Fossum GT, Davidson A,Paulson RJ. Ovarian hyperstimulation inhibits embryo implantation in the mouse. *J In Vitro Fert Embryo Transf* 1989;6:(1): 7-10.

Fowden AL, Sibley C, Reik W,Constancia M. Imprinted genes, placental development and fetal growth. *Horm Res* 2006;65 Suppl 3: 50-58.

Frank D, Fortino W, Clark L, Musalo R, Wang W, Saxena A, Li CM, Reik W, Ludwig T,Tycko B. Placental overgrowth in mice lacking the imprinted gene Ipl. *Proc Natl Acad Sci U S A* 2002;99:(11): 7490-7495.

Gicquel C, Gaston V, Mandelbaum J, Siffroi JP, Flahault A,Le Bouc Y. In vitro fertilization may increase the risk of Beckwith-Wiedemann syndrome related to the abnormal imprinting of the KCN1OT gene. *Am J Hum Genet* 2003;72:(5): 1338-1341.

Gluckman PD,Hanson MA. Maternal constraint of fetal growth and its consequences. *Semin Fetal Neonatal Med* 2004;9:(5): 419-425.

Gomes MV, Huber J, Ferriani RA, Amaral Neto AM,Ramos ES. Abnormal methylation at the KvDMR1 imprinting control region in clinically normal children conceived by assisted reproductive technologies. *Mol Hum Reprod* 2009;15:(8): 471-477.

Guillemot F, Nagy A, Auerbach A, Rossant J,Joyner AL. Essential role of Mash-2 in extraembryonic development. *Nature* 1994;371:(6495): 333-336.

Guo L, Choufani S, Ferreira J, Smith A, Chitayat D, Shuman C, Uxa R, Keating S, Kingdom J,Weksberg R. Altered gene expression and methylation of the human chromosome 11 imprinted region in small for gestational age (SGA) placentae. *Dev Biol* 2008;320:(1): 79-91.

Haig D. Genomic imprinting and kinship: how good is the evidence? *Annu Rev Genet* 2004;38: 553-585.

Halliday J, Oke K, Breheny S, Algar E,D JA. Beckwith-Wiedemann syndrome and IVF: a case-control study. *Am J Hum Genet* 2004;75:(3): 526-528.

Hansen M, Bower C, Milne E, de Klerk N,Kurinczuk JJ. Assisted reproductive technologies and the risk of birth defects--a systematic review. *Hum Reprod* 2005;20:(2): 328-338.

Hata K, Okano M, Lei H,Li E. Dnmt3L cooperates with the Dnmt3 family of de novo DNA methyltransferases to establish maternal imprints in mice. *Development* 2002;129:(8): 1983-1993.

Haycock PC,Ramsay M. Exposure of mouse embryos to ethanol during preimplantation development: effect on DNA methylation in the h19 imprinting control region. *Biol Reprod* 2009;81:(4): 618-627.

Heikkila A, Tuomisto T, Hakkinen SK, Keski-Nisula L, Heinonen S,Yla-Herttuala S. Tumor suppressor and growth regulatory genes are overexpressed in severe early-onset preeclampsia--an array study on case-specific human preeclamptic placental tissue. *Acta Obstet Gynecol Scand* 2005;84:(7): 679-689.

Helmerhorst FM, Perquin DA, Donker D,Keirse MJ. Perinatal outcome of singletons and twins after assisted conception: a systematic review of controlled studies. *Bmj* 2004;328:(7434): 261.

Hemberger M. Epigenetic landscape required for placental development. *Cell Mol Life Sci* 2007;64:(18): 2422-2436.

Herman JG,Baylin SB. Gene silencing in cancer in association with promoter hypermethylation. *N Engl J Med* 2003;349:(21): 2042-2054.

Horsthemke B,Ludwig M. Assisted reproduction: the epigenetic perspective. *Hum Reprod Update* 2005;11:(5): 473-482.

Hui P, Martel M,Parkash V. Gestational trophoblastic diseases: recent advances in histopathologic diagnosis and related genetic aspects. *Adv Anat Pathol* 2005;12:(3): 116-125.

Jackson RA, Gibson KA, Wu YW,Croughan MS. Perinatal outcomes in singletons following in vitro fertilization: a meta-analysis. *Obstet Gynecol* 2004;103:(3): 551-563.

Januchowski R, Dabrowski M, Ofori H,Jagodzinski PP. Trichostatin A down-regulate DNA methyltransferase 1 in Jurkat T cells. *Cancer Lett* 2007;246:(1-2): 313-317.

Jirtle RL,Skinner MK. Environmental epigenomics and disease susceptibility. *Nat Rev Genet* 2007;8:(4): 253-262.

Kagami M, Nagai T, Fukami M, Yamazawa K,Ogata T. Silver-Russell syndrome in a girl born after in vitro fertilization: partial hypermethylation at the differentially methylated region of PEG1/MEST. *J Assist Reprod Genet* 2007;24:(4): 131-136.

Kanayama N, Takahashi K, Matsuura T, Sugimura M, Kobayashi T, Moniwa N, Tomita M,Nakayama K. Deficiency in p57Kip2 expression induces preeclampsia-like symptoms in mice. *Mol Hum Reprod* 2002;8:(12): 1129-1135.

Kaneda M, Okano M, Hata K, Sado T, Tsujimoto N, Li E,Sasaki H. Essential role for de novo DNA methyltransferase Dnmt3a in paternal and maternal imprinting. *Nature* 2004;429:(6994): 900-903.

Kaneko-Ishino T, Kohda T,Ishino F. The regulation and biological significance of genomic imprinting in mammals. *J Biochem* 2003;133:(6): 699-711.

Katari S, Turan N, Bibikova M, Erinle O, Chalian R, Foster M, Gaughan JP, Coutifaris C,Sapienza C. DNA methylation and gene expression differences in children conceived in vitro or in vivo. *Hum Mol Genet* 2009;18:(20): 3769-3778.

Katayama S, Tomaru Y, Kasukawa T, Waki K, Nakanishi M, Nakamura M, Nishida H, Yap CC, Suzuki M, Kawai J, *et al.* Antisense transcription in the mammalian transcriptome. *Science* 2005;309:(5740): 1564-1566.

Khosla S, Dean W, Brown D, Reik W,Feil R. Culture of preimplantation mouse embryos affects fetal development and the expression of imprinted genes. *Biol Reprod* 2001;64:(3): 918-926.

Kim SJ, Park SE, Lee C, Lee SY, Kim IH, An HJ,Oh YK. Altered imprinting, promoter usage, and expression of insulin-like growth factor-II gene in gestational trophoblastic diseases. *Gynecol Oncol* 2003;88:(3): 411-418.

Kimura AP, Liebhaber SA,Cooke NE. Epigenetic modifications at the human growth hormone locus predict distinct roles for histone acetylation and methylation in placental gene activation. *Mol Endocrinol* 2004;18:(4): 1018-1032.

Koerner MV, Pauler FM, Huang R,Barlow DP. The function of non-coding RNAs in genomic imprinting. *Development* 2009;136:(11): 1771-1783.

Kono T, Obata Y, Wu Q, Niwa K, Ono Y, Yamamoto Y, Park ES, Seo JS,Ogawa H. Birth of parthenogenetic mice that can develop to adulthood. *Nature* 2004;428:(6985): 860-864.

Koslowski M, Sahin U, Mitnacht-Kraus R, Seitz G, Huber C,Tureci O. A placenta-specific gene ectopically activated in many human cancers is essentially involved in malignant cell processes. *Cancer Res* 2007;67:(19): 9528-9534.

Kovacic B,Vlaisavljevic V. Influence of atmospheric versus reduced oxygen concentration on development of human blastocysts in vitro: a prospective study on sibling oocytes. *Reprod Biomed Online* 2008;17:(2): 229-236.

Laird PW. The power and the promise of DNA methylation markers. *Nat Rev Cancer* 2003;3:(4): 253-266.

Lambertini L, Diplas AI, Lee MJ, Sperling R, Chen J,Wetmur J. A sensitive functional assay reveals frequent loss of genomic imprinting in human placenta. *Epigenetics* 2008;3:(5): 261-269.

Lee J, Inoue K, Ono R, Ogonuki N, Kohda T, Kaneko-Ishino T, Ogura A,Ishino F. Erasing genomic imprinting memory in mouse clone embryos produced from day 11.5 primordial germ cells. *Development* 2002;129:(8): 1807-1817.

Lee TI, Jenner RG, Boyer LA, Guenther MG, Levine SS, Kumar RM, Chevalier B, Johnstone SE, Cole MF, Isono K, *et al.* Control of developmental regulators by Polycomb in human embryonic stem cells. *Cell* 2006;125:(2): 301-313.

Lefebvre L, Viville S, Barton SC, Ishino F, Keverne EB,Surani MA. Abnormal maternal behaviour and growth retardation associated with loss of the imprinted gene Mest. *Nat Genet* 1998;20:(2): 163-169.

Lehnertz B, Ueda Y, Derijck AA, Braunschweig U, Perez-Burgos L, Kubicek S, Chen T, Li E, Jenuwein T,Peters AH. Suv39h-mediated histone H3 lysine 9 methylation directs DNA methylation to major satellite repeats at pericentric heterochromatin. *Curr Biol* 2003;13:(14): 1192-1200.

Lewis A, Mitsuya K, Umlauf D, Smith P, Dean W, Walter J, Higgins M, Feil R,Reik W. Imprinting on distal chromosome 7 in the placenta involves repressive histone methylation independent of DNA methylation. *Nat Genet* 2004;36:(12): 1291-1295.

Li E, Bestor TH,Jaenisch R. Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. *Cell* 1992;69:(6): 915-926.

Li HW, Tsao SW,Cheung AN. Current understandings of the molecular genetics of gestational trophoblastic diseases. *Placenta* 2002;23:(1): 20-31.

Li S, Hursting SD, Davis BJ, McLachlan JA,Barrett JC. Environmental exposure, DNA methylation, and gene regulation: lessons from diethylstilbesterol-induced cancers. *Ann N Y Acad Sci* 2003;983: 161-169.

Lie RT, Lyngstadaas A, Orstavik KH, Bakketeig LS, Jacobsen G,Tanbo T. Birth defects in children conceived by ICSI compared with children conceived by other IVF-methods; a meta-analysis. *Int J Epidemiol* 2005;34:(3): 696-701.

Lucifero D, Chaillet JR,Trasler JM. Potential significance of genomic imprinting defects for reproduction and assisted reproductive technology. *Hum Reprod Update* 2004a;10:(1): 3-18.

Lucifero D, Mann MR, Bartolomei MS,Trasler JM. Gene-specific timing and epigenetic memory in oocyte imprinting. *Hum Mol Genet* 2004b;13:(8): 839-849.

Maher ER, Brueton LA, Bowdin SC, Luharia A, Cooper W, Cole TR, Macdonald F, Sampson JR, Barratt CL, Reik W, *et al.* Beckwith-Wiedemann syndrome and assisted reproduction technology (ART). *J Med Genet* 2003;40:(1): 62-64.

Maltepe E, Krampitz GW, Okazaki KM, Red-Horse K, Mak W, Simon MC,Fisher SJ. Hypoxia-inducible factor-dependent histone deacetylase activity determines stem cell fate in the placenta. *Development* 2005;132:(15): 3393-3403.

Mancini-Dinardo D, Steele SJ, Levorse JM, Ingram RS,Tilghman SM. Elongation of the Kcnq1ot1 transcript is required for genomic imprinting of neighboring genes. *Genes Dev* 2006;20:(10): 1268-1282.

Manes C,Menzel P. Demethylation of CpG sites in DNA of early rabbit trophoblast. *Nature* 1981;293:(5833): 589-590.

Mann MR, Lee SS, Doherty AS, Verona RI, Nolen LD, Schultz RM,Bartolomei MS. Selective loss of imprinting in the placenta following preimplantation development in culture. *Development* 2004;131:(15): 3727-3735.

Market-Velker BA, Zhang L, Magri LS, Bonvissuto AC,Mann MR. Dual effects of superovulation: loss of maternal and paternal imprinted methylation in a dose-dependent manner. *Hum Mol Genet* 2010;19:(1): 36-51.

Maulik D. Fetal growth compromise: definitions, standards, and classification. *Clin Obstet Gynecol* 2006;49:(2): 214-218.

Mayer W, Niveleau A, Walter J, Fundele R,Haaf T. Demethylation of the zygotic paternal genome. *Nature* 2000;403:(6769): 501-502.

McGovern PG, Llorens AJ, Skurnick JH, Weiss G,Goldsmith LT. Increased risk of preterm birth in singleton pregnancies resulting from in vitro fertilization-embryo transfer or gamete intrafallopian transfer: a meta-analysis. *Fertil Steril* 2004;82:(6): 1514-1520.

McGrath J,Solter D. Completion of mouse embryogenesis requires both the maternal and paternal genomes. *Cell* 1984;37:(1): 179-183.

McMinn J, Wei M, Schupf N, Cusmai J, Johnson EB, Smith AC, Weksberg R, Thaker HM,Tycko B. Unbalanced placental expression of imprinted genes in human intrauterine growth restriction. *Placenta* 2006;27:(6-7): 540-549.

Meintjes M, Chantilis SJ, Douglas JD, Rodriguez AJ, Guerami AR, Bookout DM, Barnett BD,Madden JD. A controlled randomized trial evaluating the effect of lowered incubator oxygen tension on live births in a predominantly blastocyst transfer program. *Hum Reprod* 2009;24:(2): 300-307.

Miri K,Varmuza S. Imprinting and extraembryonic tissues-mom takes control. *Int Rev Cell Mol Biol* 2009;276: 215-262.

Monk D, Arnaud P, Apostolidou S, Hills FA, Kelsey G, Stanier P, Feil R,Moore GE. Limited evolutionary conservation of imprinting in the human placenta. *Proc Natl Acad Sci U S A* 2006;103:(17): 6623-6628.

Monk D,Moore GE. Intrauterine growth restriction--genetic causes and consequences. *Semin Fetal Neonatal Med* 2004;9:(5): 371-378.

Moore T,Haig D. Genomic imprinting in mammalian development: a parental tug-of-war. *Trends Genet* 1991;7:(2): 45-49.

Morison IM,Reeve AE. A catalogue of imprinted genes and parent-of-origin effects in humans and animals. *Hum Mol Genet* 1998;7:(10): 1599-1609.

Niessen HE, Demmers JA,Voncken JW. Talking to chromatin: post-translational modulation of polycomb group function. *Epigenetics Chromatin* 2009;2:(1): 10.

Novakovic B, Rakyan V, Ng HK, Manuelpillai U, Dewi C, Wong NC, Morley R, Down T, Beck S, Craig JM*, et al.* Specific tumour-associated methylation in normal human term placenta and first-trimester cytotrophoblasts. *Mol Hum Reprod* 2008;14:(9): 547-554.

Novakovic B, Sibson M, Ng HK, Manuelpillai U, Rakyan V, Down T, Beck S, Fournier T, Evain-Brion D, Dimitriadis E*, et al.* Placenta-specific methylation of the vitamin D 24-hydroxylase gene: implications for feedback autoregulation of active vitamin D levels at the fetomaternal interface. *J Biol Chem* 2009;284:(22): 14838-14848.

O'Carroll D, Erhardt S, Pagani M, Barton SC, Surani MA,Jenuwein T. The polycomb-group gene Ezh2 is required for early mouse development. *Mol Cell Biol* 2001;21:(13): 4330-4336.

Obata Y,Kono T. Maternal primary imprinting is established at a specific time for each gene throughout oocyte growth. *J Biol Chem* 2002;277:(7): 5285-5289.

Okano M, Bell DW, Haber DA,Li E. DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. *Cell* 1999;99:(3): 247-257.

Okano M, Xie S,Li E. Cloning and characterization of a family of novel mammalian DNA (cytosine-5) methyltransferases. *Nat Genet* 1998;19:(3): 219-220.

Okazaki Y, Furuno M, Kasukawa T, Adachi J, Bono H, Kondo S, Nikaido I, Osato N, Saito R, Suzuki H*, et al.* Analysis of the mouse transcriptome based on functional annotation of 60,770 full-length cDNAs. *Nature* 2002;420:(6915): 563-573.

Ono R, Nakamura K, Inoue K, Naruse M, Usami T, Wakisaka-Saito N, Hino T, Suzuki-Migishima R, Ogonuki N, Miki H*, et al.* Deletion of Peg10, an imprinted gene acquired from a retrotransposon, causes early embryonic lethality. *Nat Genet* 2006;38:(1): 101-106.

Oudejans CB, Mulders J, Lachmeijer AM, van Dijk M, Konst AA, Westerman BA, van Wijk IJ, Leegwater PA, Kato HD, Matsuda T, *et al.* The parent-of-origin effect of 10q22 in pre-eclamptic females coincides with two regions clustered for genes with down-regulated expression in androgenetic placentas. *Mol Hum Reprod* 2004;10:(8): 589-598.

Pandey RR, Mondal T, Mohammad F, Enroth S, Redrup L, Komorowski J, Nagano T, Mancini-Dinardo D,Kanduri C. Kcnq1ot1 antisense noncoding RNA mediates lineage-specific transcriptional silencing through chromatin-level regulation. *Mol Cell* 2008;32:(2): 232-246.

Pasini D, Bracken AP, Jensen MR, Lazzerini Denchi E,Helin K. Suz12 is essential for mouse development and for EZH2 histone methyltransferase activity. *Embo J* 2004;23:(20): 4061-4071.

Pauler FM, Koerner MV,Barlow DP. Silencing by imprinted noncoding RNAs: is transcription the answer? *Trends Genet* 2007;23:(6): 284-292.

Peterson CL,Laniel MA. Histones and histone modifications. *Curr Biol* 2004;14:(14): R546-551.

Pidoux G, Gerbaud P, Laurendeau I, Guibourdenche J, Bertin G, Vidaud M, Evain-Brion D,Frendo JL. Large variability of trophoblast gene expression within and between human normal term placentae. *Placenta* 2004;25:(5): 469-473.

Pineles BL, Romero R, Montenegro D, Tarca AL, Han YM, Kim YM, Draghici S, Espinoza J, Kusanovic JP, Mittal P, *et al.* Distinct subsets of microRNAs are expressed differentially in the human placentas of patients with preeclampsia. *Am J Obstet Gynecol* 2007;196:(3): 261 e261-266.

Piras G, El Kharroubi A, Kozlov S, Escalante-Alcalde D, Hernandez L, Copeland NG, Gilbert DJ, Jenkins NA,Stewart CL. Zac1 (Lot1), a potential tumor suppressor gene, and the gene for epsilon-sarcoglycan are maternally imprinted genes: identification by a subtractive screen of novel uniparental fibroblast lines. *Mol Cell Biol* 2000;20:(9): 3308-3315.

Pozharny Y, Lambertini L, Ma Y, Ferrara L, Litton CG, Diplas A, Jacobs AR, Chen J, Stone JL, Wetmur J, *et al.* Genomic loss of imprinting in first-trimester human placenta. *Am J Obstet Gynecol* 2010;202:(4): 391 e391-398.

Rahnama F, Shafiei F, Gluckman PD, Mitchell MD,Lobie PE. Epigenetic regulation of human trophoblastic cell migration and invasion. *Endocrinology* 2006;147:(11): 5275-5283.

Reik W. Stability and flexibility of epigenetic gene regulation in mammalian development. *Nature* 2007;447:(7143): 425-432.

Reik W, Constancia M, Fowden A, Anderson N, Dean W, Ferguson-Smith A, Tycko B,Sibley C. Regulation of supply and demand for maternal nutrients in mammals by imprinted genes. *J Physiol* 2003a;547:(Pt 1): 35-44.

Reik W, Dean W,Walter J. Epigenetic reprogramming in mammalian development. *Science* 2001a;293:(5532): 1089-1093.

Reik W, Santos F, Mitsuya K, Morgan H,Dean W. Epigenetic asymmetry in the mammalian zygote and early embryo: relationship to lineage commitment? *Philos Trans R Soc Lond B Biol Sci* 2003b;358:(1436): 1403-1409; discussion 1409.

Reik W,Walter J. Genomic imprinting: parental influence on the genome. *Nat Rev Genet* 2001b;2:(1): 21-32.

Renfree MB, Ager EI, Shaw G,Pask AJ. Genomic imprinting in marsupial placentation. *Reproduction* 2008;136:(5): 523-531.

Rimm AA, Katayama AC, Diaz M,Katayama KP. A meta-analysis of controlled studies comparing major malformation rates in IVF and ICSI infants with naturally conceived children. *J Assist Reprod Genet* 2004;21:(12): 437-443.

Rinaudo PF, Giritharan G, Talbi S, Dobson AT,Schultz RM. Effects of oxygen tension on gene expression in preimplantation mouse embryos. *Fertil Steril* 2006;86:(4 Suppl): 1252-1265, 1265 e1251-1236.

Rivera RM, Stein P, Weaver JR, Mager J, Schultz RM,Bartolomei MS. Manipulations of mouse embryos prior to implantation result in aberrant expression of imprinted genes on day 9.5 of development. *Hum Mol Genet* 2008;17:(1): 1-14.

Ross MG,Beall MH. Adult sequelae of intrauterine growth restriction. *Semin Perinatol* 2008;32:(3): 213-218.

Rossant J,Cross JC. Placental development: lessons from mouse mutants. *Nat Rev Genet* 2001;2:(7): 538-548.

Rossant J, Sanford JP, Chapman VM,Andrews GK. Undermethylation of structural gene sequences in extraembryonic lineages of the mouse. *Dev Biol* 1986;117:(2): 567-573.

Royo H,Cavaille J. Non-coding RNAs in imprinted gene clusters. *Biol Cell* 2008;100:(3): 149-166.

Russo VEA, Martienssen RA,Riggs AD. Epigenetic Mechanisms of Gene Regulation. *Cold Spring Harbor Laboratory Press, Woodbury* 1996.

Salas M, John R, Saxena A, Barton S, Frank D, Fitzpatrick G, Higgins MJ,Tycko B. Placental growth retardation due to loss of imprinting of Phlda2. *Mech Dev* 2004;121:(10): 1199-1210.

Santos F, Hendrich B, Reik W,Dean W. Dynamic reprogramming of DNA methylation in the early mouse embryo. *Dev Biol* 2002;241:(1): 172-182.

Santos F, Zakhartchenko V, Stojkovic M, Peters A, Jenuwein T, Wolf E, Reik W,Dean W. Epigenetic marking correlates with developmental potential in cloned bovine preimplantation embryos. *Curr Biol* 2003;13:(13): 1116-1121.

Sasaki H, Ferguson-Smith AC, Shum AS, Barton SC,Surani MA. Temporal and spatial regulation of H19 imprinting in normal and uniparental mouse embryos. *Development* 1995;121:(12): 4195-4202.

Sato A, Otsu E, Negishi H, Utsunomiya T,Arima T. Aberrant DNA methylation of imprinted loci in superovulated oocytes. *Hum Reprod* 2007;22:(1): 26-35.

Sato K, Fukata H, Kogo Y, Ohgane J, Shiota K,Mori C. Neonatal exposure to diethylstilbestrol alters expression of DNA methyltransferases and methylation of genomic DNA in the mouse uterus. *Endocr J* 2009;56:(1): 131-139.

Seely EW. Calciotropic hormones in preeclampsia: a renewal of interest. *J Clin Endocrinol Metab* 2007;92:(9): 3402-3403.

Serman L, Vlahovic M, Sijan M, Bulic-Jakus F, Serman A, Sincic N, Matijevic R, Juric-Lekic G,Katusic A. The impact of 5-azacytidine on placental weight, glycoprotein pattern and proliferating cell nuclear antigen expression in rat placenta. *Placenta* 2007;28:(8-9): 803-811.

Shin JY, Fitzpatrick GV,Higgins MJ. Two distinct mechanisms of silencing by the KvDMR1 imprinting control region. *Embo J* 2008;27:(1): 168-178.

Sibley CP, Coan PM, Ferguson-Smith AC, Dean W, Hughes J, Smith P, Reik W, Burton GJ, Fowden AL,Constancia M. Placental-specific insulin-like growth factor 2 (Igf2) regulates the diffusional exchange characteristics of the mouse placenta. *Proc Natl Acad Sci U S A* 2004;101:(21): 8204-8208.

Sims RJ, 3rd,Reinberg D. Histone H3 Lys 4 methylation: caught in a bind? *Genes Dev* 2006;20:(20): 2779-2786.

Sinclair KD, Young LE, Wilmut I,McEvoy TG. In-utero overgrowth in ruminants following embryo culture: lessons from mice and a warning to men. *Hum Reprod* 2000;15 Suppl 5: 68-86.

Singh SM, Murphy B,O'Reilly RL. Involvement of gene-diet/drug interaction in DNA methylation and its contribution to complex diseases: from cancer to schizophrenia. *Clin Genet* 2003;64:(6): 451-460.

Sleutels F, Zwart R,Barlow DP. The non-coding Air RNA is required for silencing autosomal imprinted genes. *Nature* 2002;415:(6873): 810-813.

Spahn L,Barlow DP. An ICE pattern crystallizes. *Nat Genet* 2003;35:(1): 11-12.

Steinhoff C, Paulsen M, Kielbasa S, Walter J,Vingron M. Expression profile and transcription factor binding site exploration of imprinted genes in human and mouse. *BMC Genomics* 2009;10: 144.

Surani MA, Barton SC,Norris ML. Development of reconstituted mouse eggs suggests imprinting of the genome during gametogenesis. *Nature* 1984;308:(5959): 548-550.

Swales AK,Spears N. Genomic imprinting and reproduction. *Reproduction* 2005;130:(4): 389-399.

Takahashi K, Kobayashi T,Kanayama N. p57(Kip2) regulates the proper development of labyrinthine and spongiotrophoblasts. *Mol Hum Reprod* 2000;6:(11): 1019-1025.

Tanaka M, Puchyr M, Gertsenstein M, Harpal K, Jaenisch R, Rossant J,Nagy A. Parental origin-specific expression of Mash2 is established at the time of implantation with its imprinting mechanism highly resistant to genome-wide demethylation. *Mech Dev* 1999;87:(1-2): 129-142.

Terranova R, Yokobayashi S, Stadler MB, Otte AP, van Lohuizen M, Orkin SH,Peters AH. Polycomb group proteins Ezh2 and Rnf2 direct genomic contraction and imprinted repression in early mouse embryos. *Dev Cell* 2008;15:(5): 668-679.

Thompson JG, Gardner DK, Pugh PA, McMillan WH,Tervit HR. Lamb birth weight is affected by culture system utilized during in vitro pre-elongation development of ovine embryos. *Biol Reprod* 1995;53:(6): 1385-1391.

Tierling S, Souren NY, Gries J, Loporto C, Groth M, Lutsik P, Neitzel H, Utz-Billing I, Gillessen-Kaesbach G, Kentenich H*, et al.* Assisted reproductive technologies do not enhance the variability of DNA methylation imprints in human. *J Med Genet* 2010;47:(6): 371-376.

Torres-Padilla ME. Cell identity in the preimplantation mammalian embryo: an epigenetic perspective from the mouse. *Hum Reprod* 2008;23:(6): 1246-1252.

Torres-Padilla ME, Parfitt DE, Kouzarides T,Zernicka-Goetz M. Histone arginine methylation regulates pluripotency in the early mouse embryo. *Nature* 2007;445:(7124): 214-218.

Tremblay KD, Saam JR, Ingram RS, Tilghman SM,Bartolomei MS. A paternal-specific methylation imprint marks the alleles of the mouse H19 gene. *Nat Genet* 1995;9:(4): 407-413.

Turner BM. Cellular memory and the histone code. *Cell* 2002;111:(3): 285-291.

Tycko B. Imprinted genes in placental growth and obstetric disorders. *Cytogenet Genome Res* 2006;113:(1-4): 271-278.

Umlauf D, Goto Y, Cao R, Cerqueira F, Wagschal A, Zhang Y,Feil R. Imprinting along the Kcnq1 domain on mouse chromosome 7 involves repressive histone methylation and recruitment of Polycomb group complexes. *Nat Genet* 2004;36:(12): 1296-1300.

Van der Auwera I,D'Hooghe T. Superovulation of female mice delays embryonic and fetal development. *Hum Reprod* 2001;16:(6): 1237-1243.

van Dijk M, Mulders J, Konst A, Janssens B, van Roy F, Blankenstein M,Oudejans C. Differential downregulation of alphaT-catenin expression in placenta: trophoblast cell type-dependent imprinting of the CTNNA3 gene. *Gene Expr Patterns* 2004;5:(1): 61-65.

Varmuza S,Mann M. Genomic imprinting--defusing the ovarian time bomb. *Trends Genet* 1994;10:(4): 118-123.

Vire E, Brenner C, Deplus R, Blanchon L, Fraga M, Didelot C, Morey L, Van Eynde A, Bernard D, Vanderwinden JM*, et al.* The Polycomb group protein EZH2 directly controls DNA methylation. *Nature* 2006;439:(7078): 871-874.

Vlahovic M, Bulic-Jakus F, Juric-Lekic G, Fucic A, Maric S,Serman D. Changes in the placenta and in the rat embryo caused by the demethylating agent 5-azacytidine. *Int J Dev Biol* 1999;43:(8): 843-846.

Wagschal A,Feil R. Genomic imprinting in the placenta. *Cytogenet Genome Res* 2006;113:(1-4): 90-98.

Wagschal A, Sutherland HG, Woodfine K, Henckel A, Chebli K, Schulz R, Oakey RJ, Bickmore WA,Feil R. G9a histone methyltransferase contributes to imprinting in the mouse placenta. *Mol Cell Biol* 2008;28:(3): 1104-1113.

Waldenstrom U, Engstrom AB, Hellberg D,Nilsson S. Low-oxygen compared with high-oxygen atmosphere in blastocyst culture, a prospective randomized study. *Fertil Steril* 2009;91:(6): 2461-2465.

Walsh C, Miller SJ, Flam F, Fisher RA,Ohlsson R. Paternally derived H19 is differentially expressed in malignant and nonmalignant trophoblast. *Cancer Res* 1995;55:(5): 1111-1116.

Wang J, Mager J, Chen Y, Schneider E, Cross JC, Nagy A,Magnuson T. Imprinted X inactivation maintained by a mouse Polycomb group gene. *Nat Genet* 2001;28:(4): 371-375.

Wang J, Mager J, Schnedier E,Magnuson T. The mouse PcG gene eed is required for Hox gene repression and extraembryonic development. *Mamm Genome* 2002;13:(9): 493-503.

Waterland RA,Jirtle RL. Transposable elements: targets for early nutritional effects on epigenetic gene regulation. *Mol Cell Biol* 2003;23:(15): 5293-5300.

Weaver JR, Sarkisian G, Krapp C, Mager J, Mann MR,Bartolomei MS. Domain-Specific Response of Imprinted Genes to Reduced DNMT1. *Mol Cell Biol* 2010;30:(16): 3916-3928.

Weber M,Schubeler D. Genomic patterns of DNA methylation: targets and function of an epigenetic mark. *Curr Opin Cell Biol* 2007;19:(3): 273-280.

Weisstein AE, Feldman MW,Spencer HG. Evolutionary genetic models of the ovarian time bomb hypothesis for the evolution of genomic imprinting. *Genetics* 2002;162:(1): 425-439.

Wolf JB, Cheverud JM, Roseman C,Hager R. Genome-wide analysis reveals a complex pattern of genomic imprinting in mice. *PLoS Genet* 2008;4:(6): e1000091.

Wong NC, Novakovic B, Weinrich B, Dewi C, Andronikos R, Sibson M, Macrae F, Morley R, Pertile MD, Craig JM, *et al.* Methylation of the adenomatous polyposis coli (APC) gene in human placenta and hypermethylation in choriocarcinoma cells. *Cancer Lett* 2008;268:(1): 56-62.

Wood AJ,Oakey RJ. Genomic imprinting in mammals: emerging themes and established theories. *PLoS Genet* 2006;2:(11): e147.

Wyatt SM, Kraus FT, Roh CR, Elchalal U, Nelson DM,Sadovsky Y. The correlation between sampling site and gene expression in the term human placenta. *Placenta* 2005;26:(5): 372-379.

Xue WC, Chan KY, Feng HC, Chiu PM, Ngan HY, Tsao SW,Cheung AN. Promoter hypermethylation of multiple genes in hydatidiform mole and choriocarcinoma. *J Mol Diagn* 2004;6:(4): 326-334.

Yang T, Adamson TE, Resnick JL, Leff S, Wevrick R, Francke U, Jenkins NA, Copeland NG,Brannan CI. A mouse model for Prader-Willi syndrome imprinting-centre mutations. *Nat Genet* 1998;19:(1): 25-31.

Yoder JA, Walsh CP,Bestor TH. Cytosine methylation and the ecology of intragenomic parasites. *Trends Genet* 1997;13:(8): 335-340.

Yokomine T, Kuroiwa A, Tanaka K, Tsudzuki M, Matsuda Y,Sasaki H. Sequence polymorphisms, allelic expression status and chromosome locations of the chicken IGF2 and MPR1 genes. *Cytogenet Cell Genet* 2001;93:(1-2): 109-113.

Yokomine T, Shirohzu H, Purbowasito W, Toyoda A, Iwama H, Ikeo K, Hori T, Mizuno S, Tsudzuki M, Matsuda Y, *et al.* Structural and functional analysis of a 0.5-Mb chicken region orthologous to the imprinted mammalian Ascl2/Mash2-Igf2-H19 region. *Genome Res* 2005;15:(1): 154-165.

Young LE, Fernandes K, McEvoy TG, Butterwith SC, Gutierrez CG, Carolan C, Broadbent PJ, Robinson JJ, Wilmut I,Sinclair KD. Epigenetic change in IGF2R is associated with fetal overgrowth after sheep embryo culture. *Nat Genet* 2001;27:(2): 153-154.

Yu L, Chen M, Zhao D, Yi P, Lu L, Han J, Zheng X, Zhou Y,Li L. The H19 gene imprinting in normal pregnancy and pre-eclampsia. *Placenta* 2009;30:(5): 443-447.

Zhang HJ, Siu MK, Wong ES, Wong KY, Li AS, Chan KY, Ngan HY,Cheung AN. Oct4 is epigenetically regulated by methylation in normal placenta and gestational trophoblastic disease. *Placenta* 2008;29:(6): 549-554.

Zhu XM, Han T, Sargent IL, Yin GW,Yao YQ. Differential expression profile of microRNAs in human placentas from preeclamptic pregnancies vs normal pregnancies. *Am J Obstet Gynecol* 2009;200:(6): 661 e661-667.

Chapter 5

Placentas from pregnancies conceived by IVF/ICSI have a reduced DNA methylation level at the *H19* and *MEST* differentially methylated regions

Nelissen ECM, Dumoulin JCM, Daunay A, Evers JLH, Tost J, van Montfoort APA

Human Reproduction. 2013;28(4):1117-26.

Abstract

Study question: Does IVF/ICSI have an effect on the epigenetic regulation of the human placenta?

Summary answer: We found a reduced DNA methylation level at the *H19* and *MEST* differentially methylated regions (DMRs), and an increased RNA expression of *H19* in placentas from pregnancies conceived by IVF/ICSI when compared with placentas from spontaneous conception.

What is known already: Changes in fetal environment are associated with adverse health outcomes. The placenta is pivotal for intrauterine environment. Animal studies show that epigenetic regulation plays an important role in these environment-induced phenotypic effects. Also, the preimplantation embryo environment affects birthweight as well as the risk of chronic adult diseases. Epigenetic processes are sensitive to the environment, especially during the period around conception.

Study design and participants: Placental tissue was collected from 35 spontaneously conceived pregnancies and 35 IVF/ICSI (5 IVF, 30 ICSI) derived pregnancies. We quantitatively analysed the DNA methylation patterns of a number of consecutive CpGs in the core regions of DMRs and other regulatory regions of imprinted genes, since these are involved in placental and fetal growth and development.

Methods: By using pyrosequencing, the DNA methylation at seven germline-derived primary DMRs was analysed quantitatively. Five of these are maternally methylated (*MEST* isoform α and β, *PEG3*, *KCNQ1OT1* and *SNRPN*) and two are paternally methylated [*H19* DMR and the intergenic region between *DLK1* and *MEG3* (IG-DMR)]. The post-fertilization-derived secondary DMRs, *IGF2* (DMR0 and 2) and IG-DMR (CG7, also called *MEG3* DMR), and the *MEG3* promoter region were examined as well. In case of differential methylation between the two groups, the effect on gene expression was assessed by quantitative real-time PCR.

Main results and the role of chance: Both the promoter region of *MEST* isoform α and β and the 6th CTCF binding site within the *H19* DMR were significantly hypomethylated in the IVF/ICSI group. The phenomenon was consistently observed over all CpG sites analysed and not restricted to single CpG sites. The other primary and secondary DMRs were not affected. Expression of *H19* was increased in the IVF/ICSI group, while that of *IGF2* and *MEST* remained similar.

Limitations, reasons for caution: In the IVF/ICSI group, mostly ICSI pregnancies were investigated. The ICSI technique or the male subfertility could be a confounding factor . Therefore, our results are less generalizable to IVF pregnancies.

Wider implications of the findings: The clinical effects of the observed placental hypomethylations on the developmental programming of the IVF/ICSI progeny, if any, are as yet unknown. Whether the hypomethylation is an adaptation of the placenta to maintain fetal supply and ameliorate the effects of environmental cues, or whether it is a deregulation leading to deranged developmental programming with or without increased vulnerability for disease, consistent with the developmental origins of health and disease (DOHaD) hypothesis, needs further investigation.

Study funding/competing interest(s): Partly funded by an unrestricted research grant by Organon BV (now MSD BV) without any role in study design, data collection and analysis, or preparation of the manuscript. No conflict of interests to declare.

Trial registration number: Dutch Trial Registry (NTR) number 1298

Introduction

The developmental origins of health and disease (DOHaD) hypothesis proposes that the origin of diseases manifesting in later life can be traced back to early development. This hypothesis is based on the association between disease risk and fetal and infant growth, first reported by Barker and colleagues (Barker *et al.*, 1989, Barker, 2004). The causal relationship between the mother's nutrition and the future health of her offspring confirms that the environment in the womb underlies the aetiology of diseases such as hypertension (Campbell *et al.*, 1996, Shiell *et al.*, 2001). Through a phenomenon known as "fetal programming" the fetus responds to malnutrition or other changes in the environment by permanently changing the structure of its body and adjusting its physiology and metabolism to this environment (Gluckman *et al.*, 2007). These changes put an extra burden on the organs and tissues, which, especially when there is a mismatch between the perceived intrauterine environment and the subsequent extrauterine environment, lead to chronic diseases in later life.

The mechanistic role of the placenta in the process of fetal programming is gaining attention. The size and shape of the placental surface at birth is related to the lifespan of men (Barker *et al.*, 2011) and their risk for coronary heart disease (Eriksson *et al.*, 2011). Religious fasting or a period of famine during gestation has an effect on the size and functioning of the placenta (Alwasel *et al.*, 2010, Roseboom *et al.*, 2011). The reduced efficiency creates a nutrition-deprived environment that might program the fetus towards a susceptibility for hypertension, coronary heart disease, schizophrenia and other long-term diseases reported after *in utero* exposure to famine (Kyle and Pichard, 2006).

Since genetic mutations are not indicated to underlie the modulation of programming as a reaction to the environment, it has been hypothesized that changes in the expression of genes by epigenetic regulation is involved. Among other phenomena, epigenetic processes regulate tissue-specific gene expression and expression of imprinted genes by for instance the modification of histones and/or methylation of DNA at differentially methylated regions (DMRs) (Reik and Walter, 2001). Imprinted genes are expressed in a parent-of-origin specific way and play an important role in the placenta by regulating embryonic, fetal and placental growth and development (Nelissen *et al.*, 2011a,b). The epigenetic modifications are sensitive to the environment (Jirtle and Skinner, 2007) and by nature are intensively reprogrammed during gameto- and early embryogenesis (Reik *et al.*, 2001, van Montfoort *et al.*, 2012).

Evidence emerges that not only the fetal period, but also the period around conception is highly sensitive to epigenetic programming. When rats are fed a low protein diet during the few days around conception only, the offspring had a lower birthweight and developed hypertension (Kwong *et al.*, 2000). In these rats, also a sex specific effect on the expression of the imprinted genes *H19* and *Igf2* was seen in blastocysts and fetal liver (Kwong *et al.*, 2006). Similar effects of maternal diet around conception on for instance birthweight, blood pressure, vascular function and behaviour in mice (Watkins *et al.*, 2008, Watkins *et al.*, 2010) and fetal growth, blood pressure, glucose tolerance, postnatal weight and DNA methylation in liver of sheep (MacLaughlin *et al.*, 2005, Sinclair *et al.*, 2007, Todd *et al.*, 2009) have been found.

The fact that the very early environment is important for programming might put children conceived after IVF/ICSI at risk. Indeed, a difference in birthweight was found when two different commercially available embryo culture media were used in a human IVF/ICSI program (Dumoulin *et al.*, 2010, Nelissen *et al.*, 2012). Embryos were cultured in these media for two or three days, and had a significant difference in birthweight, even after correction for several confounding factors. Further, a lower birthweight (Helmerhorst *et al.*, 2004) as well as characteristics of metabolic syndrome like vascular dysfunction, increased systolic blood pressure and fasting glucose level and skin fold thickness (Ceelen *et al.*, 2007, Ceelen *et al.*, 2008, Sakka *et al.*, 2010, Scherrer *et al.*, 2012) have been reported in human IVF/ICSI progeny, resembling the effects seen after mouse embryo culture (Fernandez-Gonzalez *et al.*, 2004, Watkins *et al.*, 2007). Little is known on the effect of IVF/ICSI on epigenetic regulation in fetal and placental tissue in human. An increased risk for an imprinting defect in IVF/ICSI offspring with Beckwith-Wiedemann or Angelman syndrome has been reported (Manipalviratn *et al.*, 2009, van Montfoort *et al.*, 2012), as well as DNA methylation differences at single CpG sites of imprinted and non-imprinted genes in placental tissue and umbilical cord blood (Gomes *et al.*, 2009, Katari *et al.*, 2009).

To investigate differences in DNA methylation in IVF/ICSI and non-IVF/ICSI derived placentas in more detail, we quantitatively analysed the DNA methylation patterns of a number of consecutive CpGs in the core regions of DMRs and other regulatory regions of imprinted genes, since these are involved in placental and fetal growth and development. Subsequently, we assessed whether differences had an effect on the expression of these genes.

Methods

Study population and sample collection

IVF/ICSI patients were recruited at the IVF centre of the Maastricht University Medical Centre (MUMC) after a positive ultrasound examination of an intact intrauterine singleton pregnancy at 7 weeks of gestation. Only pregnancies resulting from a fresh embryo transfer after standard IVF or ICSI procedures were included. IVF and ICSI treatments were performed as described previously (Dumoulin *et al.*, 2010). For all culture procedures in the current study G1™ version 5 Vitrolife medium (Göteborg, Sweden) was used.

Non-IVF/ICSI pregnant controls were recruited at the obstetric outpatients' clinic of the MUMC (34%) or at a cooperating midwife practice (66%) and were defined as having conceived spontaneously without the use of any kind of hormones, other medication or assisted reproductive treatment. Also in this group only singleton pregnancies were included. The couples were asked to fill out a short questionnaire about their height and weight before pregnancy and the smoking and drinking habits of the woman before and during pregnancy.

Within thirty minutes after delivery, placental tissue was collected by the gynaecologist, nurse, or midwife. The biopsies (~5mm^3) were taken from the fetal side, rinsed in cold, sterile PBS to remove blood and stored in RNA*later*® (Ambion, USA). Together with a copy of the delivery report, containing data on pregnancy outcome, including complications such as gestational diabetes, hypertension and pre-eclampsia, and perinatal outcome, the samples were sent to our laboratory. After receiving the samples, the amnion and chorion membranes were removed and the villi were stored until further use at -20°C according to the guidelines provided by the RNA*later*® manufacturer. All deliveries were after the 37th week of gestation.

This study was approved by the Ethical Review Board of the MUMC.

Genomic DNA extraction from placental tissue

DNA was extracted by using the Gentra PureGene tissue kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's guidelines.

Bisulfite conversion and pyrosequencing

Quantitative DNA methylation analysis was performed by pyrosequencing of bisulfite treated DNA (Tost and Gut, 2007). One µg of DNA was bisulfite converted using the EpiTect 96 Bisulfite kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's instructions. Regions of interest were amplified using 30 ng of bisulfite treated human genomic DNA and 5 - 7.5 pmol of forward and reverse primer, one of them being biotinylated. Sequences for oligonucleotides for PCR amplification and pyrosequencing are given in Table I of the supplementary data (available online). Reaction conditions were 1x HotStar Taq buffer supplemented with 1.6 mM $MgCl_2$, 100 µM dNTPs and 2.0 U HotStar Taq polymerase (Qiagen, Venlo, The Netherlands) in a 25 µl volume. The PCR program consisted of a denaturing step of 15 min at 95°C followed by 50 cycles of 30 s at 95°C, 30 s at the respective annealing temperature and 20 s at 72°C, with a final extension of 5 min at 72°C. Ten µl of PCR product were rendered single-stranded as previously described (Tost and Gut, 2007) and 4 pmol of the respective sequencing primer were used for analysis. Quantitative DNA methylation analysis was carried out on a PSQ 96MD system with the PyroGold SQA Reagent Kit (Qiagen, Venlo, The Netherlands) and results were analyzed using the Q-CpG software (V.1.0.9, Biotage AB, Uppsala Sweden).

RNA isolation from placental tissues

Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions with some minor adaptations. After adding 800 µl Trizol with 10% β-mercapto-ethanol, the samples were ground using a pestle and a mini beadbeater (Biospec, USA). RNA was precipitated with isopropyl alcohol for 2 h and the RNA pellet was washed three times with 75% ethanol. Total RNA was resuspended in 20 µl RNAse free water and stored at -80°C. RNA quantity and quality were determined using the Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, USA) and RNA integrity was measured using the Bioanalyzer 2100 (Agilent technologies, Palo Alto, USA).

cDNA synthesis

After a Dnase I treatment (Invitrogen, Carlsbad, USA), 2 µg total RNA was converted to cDNA by using the High Capacity cDNA Reverse transcription kit (Applied Biosystems, Foster City, USA) according to the manufacturer's instructions.

Quantitative Realtime-PCR (qPCR)

After cDNA synthesis, each cDNA sample (50 ng/µl) was diluted 500 times. All assays were run in triplicate in a reaction mixture containing 1 µl of the diluted sample, 5 µl reaction buffer of mesa green qPCR MasterMix plus for SYBR assay (Eurogentec, Seraing, Belgium), 1 µl of each primer ([1µM]), 1.9 µl nuclease-free water and 0.1 µl Uracil-N-Glycosylase (UNG, Eurogentec, Seraing, Belgium). The primers are listed in the supplementary data (Table SII) and were chosen to span an exon-exon barrier where possible. qRT-PCR amplification was performed using an ABI Prism 7900HT sequence detection system (Applied Biosystems, California, USA) with the following thermal cycler conditions: 2 min at 50°C for UNG treatment, 10 min at 95°C to activate Taq polymerase and 40 cycles of denaturation at 95°C for 15 s and annealing and elongation at 60°C for 1 min. A dissociation curve analysis was performed to verify the absence of genomic DNA contamination and to check the primers specificity.

The expression of *H19*, *IGF2*, *MEST*α and β was related to the expression of a combination of reference genes as described previously (van Montfoort *et al.*, 2008). Briefly, the GeNorm program (Vandesompele *et al.*, 2002) was used to determine the most stably expressed reference genes. *18S, GAPDH, YWHAZ, TBP* and *SDHA* have previously been analysed as reference genes in placental tissue (Meller *et al.*, 2005, Murthi *et al.*, 2008) and were selected for evaluation in the present study. In 22 samples, run in four different experiments, the variation in expression of *SDHA, YWHAZ* and *TBP* was the most stable. These genes were subsequently used as reference genes for the genes of interest (for primers see Table II in the supplementary data, available online). Relative expression of the genes in IVF/ICSI versus control samples was calculated using the ΔΔCt method (Livak and Schmittgen, 2001).

Statistical analysis

Statistical analysis was performed using SPSS software for windows version 16.0 (Statistical Package for Social Sciences, USA). To compare the characteristics between the IVF/ICSI and control group, Student's *t*-test was used. Birthweight SD scores (SDS) were calculated according to gestational age and gender by using the Oken reference curves as described previously (Dumoulin *et al.*, 2010, Oken *et al.*, 2003). Mean methylation, methylation per CpG site and relative gene-expression in both groups were compared by using a Mann-Whitney *U* test. The association between DNA methylation and IVF/ICSI was further

analysed by multiple linear regression analysis, while controlling for maternal age. $P < 0.05$ was considered significant.

Results

Subjects

The IVF/ICSI children were conceived after conventional IVF (n=5) or ICSI (n=30). The indications for IVF/ICSI were male factor (n=28), tubal factor (n=2), treatment-resistant polycystic ovary syndrome (n=1) and unexplained subfertility (n=4). In the control and IVF/ICSI group 14 and 18 newborns were male and 21 and 17 female, respectively. All were singletons born after 37 weeks of gestation. The characteristics of the parents were similar in both groups, except for the older age in the IVF/ICSI group. Regarding the pregnancy complications, there were none in the control group and two (1 hypertension and 1 HELLP syndrome (characterised by haemolysis, elevated liver enzymes and low platelets)) in the IVF/ICSI group (not significant, Fisher's Exact test).

DNA Methylation at different DMRs in placental tissue

Seven germline-derived DMRs were analysed of which 5 are maternally methylated (*MEST* (Mesoderm-specific transcript, a.k.a. paternally expressed gene 1 (PEG1)) isoform α and β, *PEG3*, *KCNQ1OT1* and *SNRPN*) and 2 are paternally methylated (*H19* DMR and the intergenic region between *DLK1* and *MEG3* (IG-DMR)). Within the *H19* DMR (also known as H19/IGF2 ICR1) we analysed the 3th and the 6th CTCF binding site and within IG-DMR two CpG islands, CG4 and 6, were analysed (Kagami *et al.*, 2008, Kagami *et al.*, 2010). Further the post-fertilization-derived secondary DMRs in *IGF2* (DMR0 and 2) and IG-DMR (CG7, also called *MEG3* DMR), and the *MEG3* promoter region were subjected to pyrosequencing.

In Figure I the average methylation of the analysed regions is depicted for the IVF/ICSI and control group. The location of these regions within chromosomal context is shown in Figure II. IVF/ICSI placentas were relatively hypomethylated in the CTCF6 region of *H19* DMR and the promoter regions of *MEST* isoform α and β and *MEG3* (Figure I). For both *MEST* isoforms, maternal age was no confounder, since after adjustment for maternal age by linear regression analysis the group was still significantly associated with DNA methylation ($P<0.05$). When using the average methylation of the *H19* CTCF6 region, the association between

group and DNA methylation disappeared after adjustment for age. However, when using the average methylation of the differentially methylated CpGs only (see Figure II), the association remained significant ($P<0.05$). *MEG3* promoter methylation was maternal age related, and therewith not significantly different after correction. In the other regions, the methylation level was similar in both groups, although often slightly lower in the IVF/ICSI group. Methylation levels between the DMRs ranged from 27-64%, suggesting a different distribution of cells with non-, mono- or biallelic methylation for each DMR. The variation from the average level of methylation differed significantly in 9 out of the 14 analysed regions (Mann-Whitney *U* test). In all these regions (*IGF2 DMR 2.2, IGF2 DMR 2.1, H19 CTCF3, IG-DMR CG6, MEG3, MEST* α and β, *PEG3* and *KCNQ1OT1*), the variation was lowest in the IVF/ICSI group.

One sample in the control group had outlying high methylation levels for maternally methylated *MEST* isoform β (88.1%), *PEG3* (61.5%) and *KCNQ1OT1* (86.3%), and outlying low methylation levels for *SNRPN* (9.6%). The methylation level of the paternally methylated regions was within the range of the other samples. This sample had hardly any effect on the comparison between IVF/ICSI and control samples, since after excluding this sample, the difference in DNA methylation at *H19* DMR and both *MEST* DMRs was still significant. The difference in variation between both groups disappeared for *MEST β* and *PEG3*, and became lower but still significant for *KCNQ1OT1* when this sample was excluded. In this child, no congenital malformations were reported at birth.

The methylation level per CpG within the DMRs analysed is shown in Figure II. Within the 6th CTCF binding region of *H19* DMR (Figure IIA) the methylation level in the IVF/ICSI group is systematically lower per CpG. For all the other paternally methylated CpGs, methylation was similar. The methylation level of the secondary DMR CG7 (*MEG3* DMR) is lower compared with the other analysed primary CpG regions within IG-DMR, but at the same level as the *MEG3* promoter, which is also methylated post-fertilization.

Regarding the maternally methylated DMRs (Figure IIB), the methylation level of all CpGs within both isoforms of *MEST* are hypomethylated in the IVF/ICSI group (most, but not all significant). In both groups, the methylation level of the α isoform is higher than that of the placenta specific β form. For the other DMRs, methylation levels at all CpGs are similar in both groups. The methylation of *KCNQ1OT1* seems distinct in both groups, but as the variation in the control group (described above) is relatively high, this does not reach statistical significance.

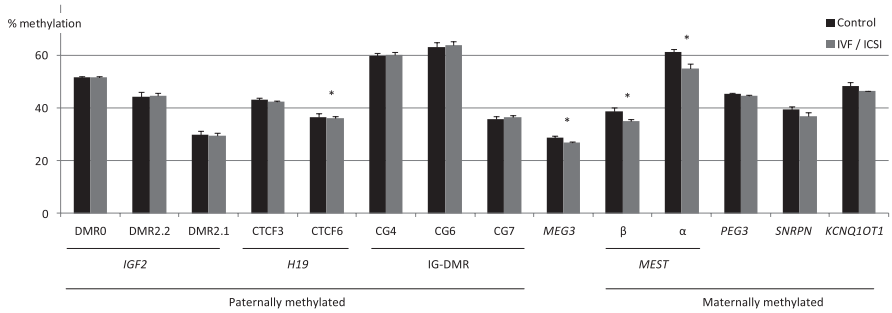
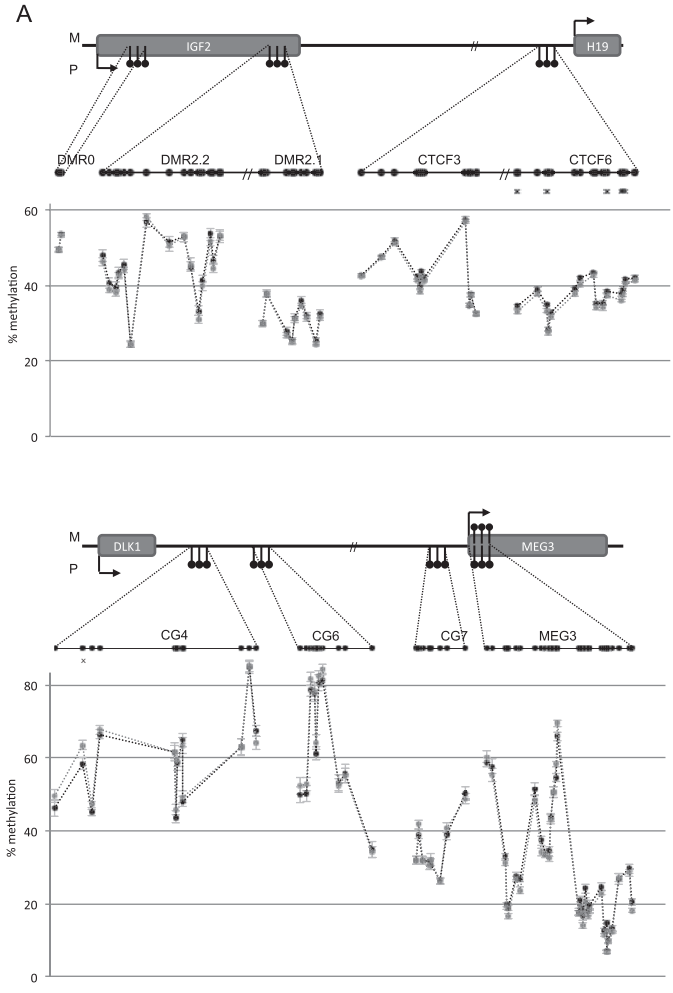


Figure I Average methylation (and SEM) of the analysed regions in placental tissue obtained from naturally conceived (control; n=35) and IVF/ICSI conceived (IVF/ICSI; n=35) pregnancies. All regions, except MEG3, are known to be methylated in a parent-of-origin specific manner. The location of the regions in relation to the genes is shown in Figure 2. DMR, differentially methylated region; CTCF, CTCF binding site; CG, CpG island; IG-DMR, intergenic region between DLK1 and MEG3; β and α, isoforms of MEST. * P<0.05 (Mann-Whitney U-test).



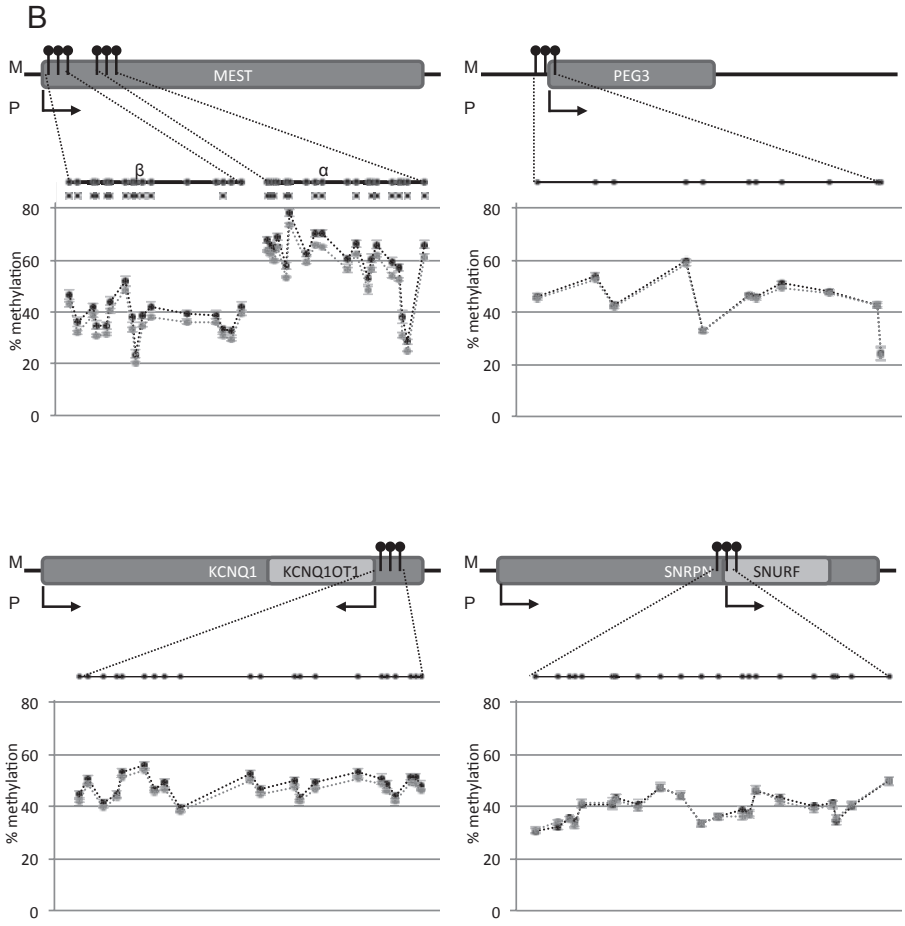


Figure 2 Average methylation per CpG within the analysed regions in placental tissue obtained from naturally conceived (control; n=35) and IVF/ICSI conceived (IVF/ICSI; n=35) pregnancies. (**A**) Paternally imprinted regions and MEG3, (**B**) maternally imprinted regions. In the upper parts, the genes with their transcription start sites (arrows) and the location of the analysed methylated regions (lollipops) are depicted (not on scale). The position of the lollipops and the arrows indicates the parental methylation and expression, respectively (P, paternal; M, maternal). In the upper part of the graphs, the analysed CpGs (black circles) and their relative position are indicated. The methylation level at these CpGs in the control (black) and the IVF/ICSI (grey) group is expressed as mean percentage ± SEM. For the sake of clarity, a dashed line is drawn between the circles. The CpGs that are marked with 'x' differ in methylation between IVF/ICSI and control, $P < 0.05$, Mann-Whitney *U*-test.

Gene expression of H19 and MEST in placental tissue

Since *MEST* and *H19* DMR are significantly hypomethylated in the IVF/ICSI group, we assessed whether this has an effect on the gene expression of the DMR-regulated genes. Expression of both *MEST* isoforms was not affected, neither was the expression of *IGF2*. Only *H19* showed a significant change, a 1.3 fold increase in expression in the IVF/ICSI group (Figure III).

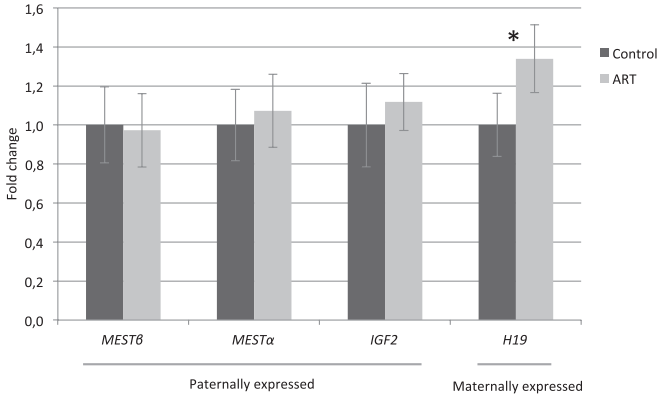


Figure III Relative gene expression of the two *MEST* isoforms, *IGF2* and *H19* in placental tissue derived from spontaneous pregnancies (control; n=35) and IVF/ICSI pregnancies (IVF/ICSI; n=35). Control group is set at one and IVF/ICSI is related to the control. Error bars represent SEM. * $P < 0.05$

Neonatal characteristics

Mean birthweight and birthweight SDS ± SD were 3386g ± 516 and -0.23 ± 1.0 for the control group and 3378g ± 445 and -0.22 ± 0.9 for the IVF/ICSI group, respectively (not significant). The mean gestational age was 39.8 weeks in both groups. The hypomethylation of *H19* DMR and *MEST* and the increased expression of *H19* are not linearly related to changes in birthweight, birthweight SDS or gestational age as assessed by linear regression analysis.

Discussion

From the seven analysed DMRs of imprinted genes, the primary paternally methylated *H19* DMR (CTCF6) and maternally methylated *MEST* α and β promoter were relatively hypomethylated in IVF/ICSI derived placentas compared with placentas from spontaneous conception. Both the average methylation level of the analysed region as well as the methylation level of most of the individual CpGs differed significantly. The RNA expression of *H19* was increased in the IVF/ICSI group, while *MEST* was similarly expressed in both groups. Secondary imprint regions were not affected.

The specific methylation pattern for each DMR is maintained for each sample, and also between the control and IVF/ICSI group, notwithstanding the lower level for some DMRs. This suggests a strict regulation of DNA methylation. The variation in methylation significantly differs in several of the regions analysed, and was lower in the IVF/ICSI group. This is in contrast to what was reported by Turan *et al.* (2010), who found a higher variation in DNA methylation in IVF placental tissue. The higher variation evolved out of a lower number of trophoblast stem cells from which the IVF placenta was derived. This was calculated using the distribution of methylation scores of two different regions (X chromosome and *H19* DMR) in 5 sections of each placenta from all the individuals in the two groups. To conclude that in our study the lower variation in the IVF/ICSI group originates from a higher number of trophoblast stem cells is a bit premature, considering our confined analysis. However, on the other hand it seems unlikely that if there would be a higher variation in DNA methylation within our IVF/ICSI group, by chance only those samples are selected that regarding the level of DNA methylation are closely related to each other.

The hypomethylation of the *MEST* DMR in the IVF/ICSI group corresponds to the hypomethylation reported after IVF in nine single CpGs within this DMR in placental tissue (Rancourt *et al.*, 2012) and in two single CpGs within this DMR in both placental tissue and umbilical cord blood (Katari *et al.*, 2009). In another study, in which the majority of IVF pregnancies concerned twins, no difference was reported (Tierling *et al.*, 2010). The two isoforms of *MEST* differ with respect to the tissue-specific imprinting. The long downstream isoform α is imprinted in all tissues, while shorter upstream isoform β is not imprinted in several non-placental tissues. McMinn *et al.* (2006) reported that isoform β is imprinted in

the placenta, but that the inter-individual variation in DNA methylation of the maternal allele was larger than that of isoform α or other imprinted genes. This is in contrast with our data where the variation is similar in both *MEST* regions, although the methylation level in the CpG island of the short isoform (β) is lower than that of the longer α isoform. Further, it is interesting to note that *Mest* is methylated rapidly, only at the very late stage of oogenesis (late antral follicle stage), when ovarian stimulation takes place, in contrast to *Snrpn* and *Peg3* where methylation starts already at the early growing stage (pre-antral follicles) (Lucifero *et al.*, 2002).

The hypomethylation of the *H19* DMR is region specific, in that only the *IGF2* and *H19* expression regulating domain CTCF6 is affected (Takai *et al.*, 2001) and not CTCF3, which might explain the conflicting data reported so far in IVF derived placental tissue [hypomethylation (Rancourt *et al.*, 2012, Turan *et al.*, 2010); no difference (Katari *et al.*, 2009, Tierling *et al.*, 2010, Wong *et al.*, 2011)]. In human preimplantation IVF embryos, a hypomethylation of *H19* DMR has been reported in 18.7% of the embryos (Chen *et al.*, 2010)2010. Even in good-quality cryopreserved blastocysts donated for research, a lower-than-expected methylation level (when compared with methylation in sperm samples from fertile men) was reported (Ibala-Romdhane *et al.*, 2011), suggesting that *H19* is a labile region. In mice studies, designed to validate epigenetic regulation in response to IVF/ICSI, *H19* is most frequently involved. After ovulation induction (Fortier *et al.*, 2008) or embryo culture (Mann *et al.*, 2004, Rivera *et al.*, 2008) a loss of methylation and/or an increase in biallelic expression was found specifically in the placenta, while fetal tissues were not affected.

The cause of the reported hypomethylation in *MEST* and *H19* DMR might be found in the IVF/ICSI technique or in an intrinsic heritable factor of the subfertile patients. Since our population consisted mainly of male subfertile patients applying for ICSI, an intrinsic male factor might play a role. However, an incomplete remethylation in one of the two gametes would result in a complete demethylation in all cells and not in the hypomethylated pattern that we see now. Although hypomethylation is often reported in spermatozoa from oligozoospermic patients, transmission of these defects to viable offspring has never been reported (van Montfoort *et al.*, 2012). The transmittance via a paternal or maternal cellular effect (i.e. regulatory RNAs) is possible, although they mainly seem to affect transposons and other repeat sequences (Daxinger and Whitelaw, 2012). In sperm from mice on a low-protein diet transmitting

hepatic effects to their offspring, differences in RNAs and chromatin packaging have been identified (Carone *et al.*, 2010). Effects on imprinted genes were, however, not reported. Therefore, at the moment, the reduced methylation is most likely due to a lack of maintenance of methylation after fertilization, and thus the IVF/ICSI technique itself. The culture medium in combination with the hormonally primed uterus might play a role. In mice, transferring an embryo from a hormonally primed to a non-stimulated uterus reduced the ovarian stimulation effects on imprinting (Fortier *et al.*, 2008). Culture medium has been extensively shown to affect epigenetic regulation in several animal species (Fauque *et al.*, 2010a,b, Khosla *et al.*, 2001, Market-Velker *et al.*, 2010, Young *et al.*, 2001). In the human, this has not been directly investigated, although an effect of culture medium on birthweight (Dumoulin *et al.*, 2010, Nelissen *et al.*, 2012) and on the placental production of free-βhCG at 12 weeks of gestation has been reported (Nelissen *et al.*, 2011b). Also an effect of the ICSI technique per se cannot be ruled out since our IVF/ICSI population mainly consists of ICSI pregnancies. However, we noted no difference in methylation between IVF and ICSI although IVF numbers were very small (data not shown), there is no convincing evidence that ICSI elevates the risk of epigenetic abnormalities when compared with IVF, and imprinting disorders occur after both IVF and ICSI (van Montfoort *et al.*, 2012).

The level of the reduction in methylation found in this study is consistent with the hypo- or hypermethylation levels reported after other periconception changes of environment. People exposed periconceptionally to the Dutch famine show significant changes in DNA methylation, ranging from 0.5 to 5%, >55 years later (Heijmans *et al.*, 2008, Tobi *et al.*, 2009). In ewes, restricting the supply of B vitamins in the periconceptional diet led to a 4% reduction of overall methylation in the offspring, together with an increase in body weight, insulin resistance and blood pressure (Sinclair *et al.*, 2007). The culture of mouse embryos in different culture media led to methylation differences in blastocysts ranging from a few percent to >10% (Fauque *et al.*, 2010a,b, Market-Velker *et al.*, 2010).

The clinical effects of the observed placental hypomethylations on the developmental programming of the IVF/ICSI progeny, if any, are as yet unknown. The altered *MEST* methylation did not affect *MEST* expression of the corresponding isoform, suggesting that either a certain threshold has to be overcome or equally likely, that other epigenetic regulatory mechanisms like

histone modifications are involved as well (Riclet *et al.*, 2009). *Mest* deficient mice show a reduced fetal growth, a smaller placental size (Lefebvre *et al.*, 1998) and an impaired angiogenesis (Mayer *et al.*, 2000). No reports exist on long-term outcome of *Mest*-reduced placentas. *H19* is widely expressed in the placenta. A deletion in mice leads to placentomegaly and fetal overgrowth resulting from a *cis* effect on loss of imprinting and biallelic expression of *Igf2* (Eggenschwiler *et al.*, 1997). Placental efficiency, as well as placental permeability, is reduced, suggesting that growth-control adaptations are made in order to reduce nutrient supply to the overgrowing fetus (Fowden *et al.*, 2011). The degree of hypomethylation in our samples was accompanied by an overexpression of *H19,* but no effect on *IGF2* expression or fetal growth was found, in contrast to what is reported in mice (Gabory *et al.*, 2009).

The clinical significance of our findings remains unknown. Whether the hypomethylation is an adaptation of the placenta to maintain fetal supply and ameliorate the effects of environmental cues, or whether it is a deregulation leading to deranged developmental programming with or without increased vulnerability for disease, consistent with the DOHaD hypothesis, needs further investigation.

Acknowledgements

The authors want to thank the midwifes Patricia Bemelmans, Chantal Mulkens, Charlotte Peerboom and Ellen de Jong from the midwife practice Sittard, together with the midwifes from several practices surrounding Maastricht and the gynaecologists and residents gynaecology and obstetrics from the Atrium hospital Heerlen and Orbis hospital Sittard for their assistance in collecting placental tissues. Marijke Bras, Inge Schreurs, Sander Kleijkers and Roel Thijssen are thanked for their assistance in processing the samples. Peter de Boer is acknowledged for critical consideration of the manuscript.

References

Alwasel, S. H., Abotalib, Z., Aljarallah, J. S., Osmond, C., Alkharaz, S. M., Alhazza, I. M., Badr, G. and Barker, D. J. Changes in placental size during Ramadan. *Placenta* 2010;**31:**607-610.

Barker, D. J., Osmond, C., Golding, J., Kuh, D. and Wadsworth, M. E. Growth in utero, blood pressure in childhood and adult life, and mortality from cardiovascular disease. *Bmj* 1989;**298:**564-567.

Barker, D. J. The developmental origins of adult disease. *J Am Coll Nutr* 2004;**23:**588S-595S.

Barker, D. J., Osmond, C., Thornburg, K. L., Kajantie, E. and Eriksson, J. G. The lifespan of men and the shape of their placental surface at birth. *Placenta* 2011;**32:**783-787.

Campbell, D. M., Hall, M. H., Barker, D. J., Cross, J., Shiell, A. W. and Godfrey, K. M. Diet in pregnancy and the offspring's blood pressure 40 years later. *Br J Obstet Gynaecol* 1996;**103:**273-280.

Carone, B. R., Fauquier, L., Habib, N., Shea, J. M., Hart, C. E., Li, R., Bock, C., Li, C., Gu, H., Zamore, P. D. et al. Paternally induced transgenerational environmental reprogramming of metabolic gene expression in mammals. *Cell* 2010;**143:**1084-1096.

Ceelen, M., van Weissenbruch, M. M., Roos, J. C., Vermeiden, J. P., van Leeuwen, F. E. and Delemarre-van de Waal, H. A. Body composition in children and adolescents born after in vitro fertilization or spontaneous conception. *J Clin Endocrinol Metab* 2007;**92:**3417-3423.

Ceelen, M., van Weissenbruch, M. M., Vermeiden, J. P., van Leeuwen, F. E. and Delemarre-van de Waal, H. A. Cardiometabolic differences in children born after in vitro fertilization: follow-up study. *J Clin Endocrinol Metab* 2008;**93:**1682-1688.

Chen, S. L., Shi, X. Y., Zheng, H. Y., Wu, F. R. and Luo, C. Aberrant DNA methylation of imprinted H19 gene in human preimplantation embryos. *Fertil Steril* 2010;**94:**2356-2358.

Daxinger, L. and Whitelaw, E. Understanding transgenerational epigenetic inheritance via the gametes in mammals. *Nature reviews Genetics* 2012;**13:**153-162.

Dumoulin, J. C., Land, J. A., Van Montfoort, A. P., Nelissen, E. C., Coonen, E., Derhaag, J. G., Schreurs, I. L., Dunselman, G. A., Kester, A. D., Geraedts, J. P. et al. Effect of in vitro culture of human embryos on birthweight of newborns. *Hum Reprod* 2010;**25:**605-612.

Eggenschwiler, J., Ludwig, T., Fisher, P., Leighton, P. A., Tilghman, S. M. and Efstratiadis, A. Mouse mutant embryos overexpressing IGF-II exhibit phenotypic features of the Beckwith-Wiedemann and Simpson-Golabi-Behmel syndromes. *Genes Dev* 1997;**11:**3128-3142.

Eriksson, J. G., Kajantie, E., Thornburg, K. L., Osmond, C. and Barker, D. J. Mother's body size and placental size predict coronary heart disease in men. *Eur Heart J* 2011;**32:**2297-2303.

Fauque, P., Mondon, F., Letourneur, F., Ripoche, M. A., Journot, L., Barbaux, S., Dandolo, L., Patrat, C., Wolf, J. P., Jouannet, P. et al. In vitro fertilization and embryo culture strongly impact the placental transcriptome in the mouse model. *PLoS One* 2010a;**5:**e9218.

Fauque, P., Ripoche, M. A., Tost, J., Journot, L., Gabory, A., Busato, F., Le Digarcher, A., Mondon, F., Gut, I., Jouannet, P. et al. Modulation of imprinted gene network in placenta results in normal development of in vitro manipulated mouse embryos. *Human molecular genetics* 2010b;**19:**1779-1790.

Fernandez-Gonzalez, R., Moreira, P., Bilbao, A., Jimenez, A., Perez-Crespo, M., Ramirez, M. A., Rodriguez De Fonseca, F., Pintado, B. and Gutierrez-Adan, A. Long-term effect of in vitro culture of mouse embryos with serum on mRNA expression of imprinting genes, development, and behavior. *Proc Natl Acad Sci U S A* 2004;**101:**5880-5885.

Fortier, A. L., Lopes, F. L., Darricarrere, N., Martel, J. and Trasler, J. M. Superovulation alters the expression of imprinted genes in the midgestation mouse placenta. *Human molecular genetics* 2008;**17:**1653-1665.

Fowden, A. L., Coan, P. M., Angiolini, E., Burton, G. J. and Constancia, M. Imprinted genes and the epigenetic regulation of placental phenotype. *Prog Biophys Mol Biol* 2011;**106:**281-288.

Gabory, A., Ripoche, M. A., Le Digarcher, A., Watrin, F., Ziyyat, A., Forne, T., Jammes, H., Ainscough, J. F., Surani, M. A., Journot, L. et al. H19 acts as a trans regulator of the imprinted gene network controlling growth in mice. *Development* 2009;**136:**3413-3421.

Gluckman, P. D., Hanson, M. A. and Beedle, A. S. Early life events and their consequences for later disease: a life history and evolutionary perspective. *Am J Hum Biol* 2007;**19:**1-19.

Gomes, M. V., Huber, J., Ferriani, R. A., Amaral Neto, A. M. and Ramos, E. S. Abnormal methylation at the KvDMR1 imprinting control region in clinically normal children conceived by assisted reproductive technologies. *Mol Hum Reprod* 2009;**15:**471-477.

Heijmans, B. T., Tobi, E. W., Stein, A. D., Putter, H., Blauw, G. J., Susser, E. S., Slagboom, P. E. and Lumey, L. H. Persistent epigenetic differences associated with prenatal exposure to famine in humans. *Proc Natl Acad Sci U S A* 2008;**105:**17046-17049.

Helmerhorst, F. M., Perquin, D. A., Donker, D. and Keirse, M. J. Perinatal outcome of singletons and twins after assisted conception: a systematic review of controlled studies. *Bmj* 2004;**328:**261.

Ibala-Romdhane, S., Al-Khtib, M., Khoueiry, R., Blachere, T., Guerin, J. F. and Lefevre, A. Analysis of H19 methylation in control and abnormal human embryos, sperm and oocytes. *Eur J Hum Genet* 2011;**19:**1138-1143.

Jirtle, R. L. and Skinner, M. K. Environmental epigenomics and disease susceptibility. *Nature reviews Genetics* 2007;**8:**253-262.

Kagami, M., Sekita, Y., Nishimura, G., Irie, M., Kato, F., Okada, M., Yamamori, S., Kishimoto, H., Nakayama, M., Tanaka, Y. et al. Deletions and epimutations affecting the human 14q32.2 imprinted region in individuals with paternal and maternal upd(14)-like phenotypes. *Nat Genet* 2008;**40:**237-242.

Kagami, M., O'Sullivan, M. J., Green, A. J., Watabe, Y., Arisaka, O., Masawa, N., Matsuoka, K., Fukami, M., Matsubara, K., Kato, F. et al. The IG-DMR and the MEG3-DMR at human chromosome 14q32.2: hierarchical interaction and distinct functional properties as imprinting control centers. *PLoS Genet* 2010;**6:**e1000992.

Katari, S., Turan, N., Bibikova, M., Erinle, O., Chalian, R., Foster, M., Gaughan, J. P., Coutifaris, C. and Sapienza, C. DNA methylation and gene expression differences in children conceived in vitro or in vivo. *Human molecular genetics* 2009;**18:**3769-3778.

Khosla, S., Dean, W., Brown, D., Reik, W. and Feil, R. Culture of preimplantation mouse embryos affects fetal development and the expression of imprinted genes. *Biology of reproduction* 2001;**64:**918-926.

Kwong, W. Y., Wild, A. E., Roberts, P., Willis, A. C. and Fleming, T. P. Maternal undernutrition during the preimplantation period of rat development causes blastocyst abnormalities and programming of postnatal hypertension. *Development* 2000;**127:**4195-4202.

Kwong, W. Y., Miller, D. J., Ursell, E., Wild, A. E., Wilkins, A. P., Osmond, C., Anthony, F. W. and Fleming, T. P. Imprinted gene expression in the rat embryo-fetal axis is altered in response to periconceptional maternal low protein diet. *Reproduction* 2006;**132:**265-277.

Kyle, U. G. and Pichard, C. The Dutch Famine of 1944-1945: a pathophysiological model of long-term consequences of wasting disease. *Curr Opin Clin Nutr Metab Care* 2006;**9:**388-394.

Lefebvre, L., Viville, S., Barton, S. C., Ishino, F., Keverne, E. B. and Surani, M. A. Abnormal maternal behaviour and growth retardation associated with loss of the imprinted gene Mest. *Nat Genet* 1998;**20:**163-169.

Livak, K. J. and Schmittgen, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001;**25:**402-408.

Lucifero, D., Mertineit, C., Clarke, H. J., Bestor, T. H. and Trasler, J. M. Methylation dynamics of imprinted genes in mouse germ cells. *Genomics* 2002;**79:**530-538.

MacLaughlin, S. M., Walker, S. K., Roberts, C. T., Kleemann, D. O. and McMillen, I. C. Periconceptional nutrition and the relationship between maternal body weight changes in the periconceptional period and feto-placental growth in the sheep. *J Physiol* 2005;**565:**111-124.

Manipalviratn, S., DeCherney, A. and Segars, J. Imprinting disorders and assisted reproductive technology. *Fertil Steril* 2009;**91:**305-315.

Mann, M. R., Lee, S. S., Doherty, A. S., Verona, R. I., Nolen, L. D., Schultz, R. M. and Bartolomei, M. S. Selective loss of imprinting in the placenta following preimplantation development in culture. *Development* 2004;**131:**3727-3735.

Market-Velker, B. A., Fernandes, A. D. and Mann, M. R. Side-by-side comparison of five commercial media systems in a mouse model: suboptimal in vitro culture interferes with imprint maintenance. *Biology of reproduction* 2010;**83:**938-950.

Mayer, W., Hemberger, M., Frank, H. G., Grummer, R., Winterhager, E., Kaufmann, P. and Fundele, R. Expression of the imprinted genes MEST/Mest in human and murine placenta suggests a role in angiogenesis. *Dev Dyn* 2000;**217:**1-10.

McMinn, J., Wei, M., Sadovsky, Y., Thaker, H. M. and Tycko, B. Imprinting of PEG1/MEST isoform 2 in human placenta. *Placenta* 2006;**27:**119-126.

Meller, M., Vadachkoria, S., Luthy, D. A. and Williams, M. A. Evaluation of housekeeping genes in placental comparative expression studies. *Placenta* 2005;**26:**601-607.

Murthi, P., Fitzpatrick, E., Borg, A. J., Donath, S., Brennecke, S. P. and Kalionis, B. GAPDH, 18S rRNA and YWHAZ are suitable endogenous reference genes for relative gene expression studies in placental tissues from human idiopathic fetal growth restriction. *Placenta* 2008;**29:**798-801.

Nelissen, E. C., van Montfoort, A. P., Dumoulin, J. C. and Evers, J. L. Epigenetics and the placenta. *Human reproduction update* 2011b;**17:**397-417.

Nelissen, E. C., Van Montfoort, A. P., Coonen, E., Derhaag, J. G., Geraedts, J. P., Smits, L. J., Land, J. A., Evers, J. L. and Dumoulin, J. C. Further evidence that culture media affect perinatal outcome: findings after transfer of fresh and cryopreserved embryos. *Hum Reprod* 2012;**27:**1966-1976.

Nelissen, E. C. M., van Montfoort, A. P. A., Menheere, P. P. C. A., Evers, J. L. H., Peeters, L. L. and Dumoulin, J. C. M. Effect of IVF culture medium on human fetal growth. *Hum Reprod* 2011a;**26(suppl1):**i67.

Oken, E., Kleinman, K. P., Rich-Edwards, J. and Gillman, M. W. A nearly continuous measure of birth weight for gestational age using a United States national reference. *BMC Pediatr* 2003;**3:**6.

Rancourt, R. C., Harris, H. R. and Michels, K. B. Methylation levels at imprinting control regions are not altered with ovulation induction or in vitro fertilization in a birth cohort. *Hum Reprod* 2012;**27:**2208-2216.

Reik, W., Dean, W. and Walter, J. Epigenetic reprogramming in mammalian development. *Science* 2001;**293:**1089-1093.

Reik, W. and Walter, J. Genomic imprinting: parental influence on the genome. *Nature reviews Genetics* 2001;**2:**21-32.

Riclet, R., Chendeb, M., Vonesch, J. L., Koczan, D., Thiesen, H. J., Losson, R. and Cammas, F. Disruption of the interaction between transcriptional intermediary factor 1{beta} and heterochromatin protein 1 leads to a switch from DNA hyper- to hypomethylation and H3K9 to H3K27 trimethylation on the MEST promoter correlating with gene reactivation. *Mol Biol Cell* 2009;**20:**296-305.

Rivera, R. M., Stein, P., Weaver, J. R., Mager, J., Schultz, R. M. and Bartolomei, M. S. Manipulations of mouse embryos prior to implantation result in aberrant expression of imprinted genes on day 9.5 of development. *Human molecular genetics* 2008;**17:**1-14.

Roseboom, T. J., Painter, R. C., de Rooij, S. R., van Abeelen, A. F., Veenendaal, M. V., Osmond, C. and Barker, D. J. Effects of famine on placental size and efficiency. *Placenta* 2011;**32:**395-399.

Sakka, S. D., Loutradis, D., Kanaka-Gantenbein, C., Margeli, A., Papastamataki, M., Papassotiriou, I. and Chrousos, G. P. Absence of insulin resistance and low-grade inflammation despite early metabolic syndrome manifestations in children born after in vitro fertilization. *Fertil Steril* 2010;**94:**1693-1699.

Scherrer, U., Rimoldi, S. F., Rexhaj, E., Stuber, T., Duplain, H., Garcin, S., de Marchi, S. F., Nicod, P., Germond, M., Allemann, Y. et al. Systemic and pulmonary vascular dysfunction in children conceived by assisted reproductive technologies. *Circulation* 2012;**125:**1890-1896.

Shiell, A. W., Campbell-Brown, M., Haselden, S., Robinson, S., Godfrey, K. M. and Barker, D. J. High-meat, low-carbohydrate diet in pregnancy: relation to adult blood pressure in the offspring. *Hypertension* 2001;**38:**1282-1288.

Sinclair, K. D., Allegrucci, C., Singh, R., Gardner, D. S., Sebastian, S., Bispham, J., Thurston, A., Huntley, J. F., Rees, W. D., Maloney, C. A. et al. DNA methylation, insulin resistance, and blood pressure in offspring determined by maternal periconceptional B vitamin and methionine status. *Proc Natl Acad Sci U S A* 2007;**104:**19351-19356.

Takai, D., Gonzales, F. A., Tsai, Y. C., Thayer, M. J. and Jones, P. A. Large scale mapping of methylcytosines in CTCF-binding sites in the human H19 promoter and aberrant hypomethylation in human bladder cancer. *Human molecular genetics* 2001;**10:**2619-2626.

Tierling, S., Souren, N. Y., Gries, J., Loporto, C., Groth, M., Lutsik, P., Neitzel, H., Utz-Billing, I., Gillessen-Kaesbach, G., Kentenich, H. et al. Assisted reproductive technologies do not enhance the variability of DNA methylation imprints in human. *J Med Genet* 2010;**47:**371-376.

Tobi, E. W., Lumey, L. H., Talens, R. P., Kremer, D., Putter, H., Stein, A. D., Slagboom, P. E. and Heijmans, B. T. DNA methylation differences after exposure to prenatal famine are common and timing- and sex-specific. *Human molecular genetics* 2009;**18:**4046-4053.

Todd, S. E., Oliver, M. H., Jaquiery, A. L., Bloomfield, F. H. and Harding, J. E. Periconceptional undernutrition of ewes impairs glucose tolerance in their adult offspring. *Pediatr Res* 2009;**65:**409-413.

Tost, J. and Gut, I. G. DNA methylation analysis by pyrosequencing. *Nat Protoc* 2007;**2:**2265-2275.

Turan, N., Katari, S., Gerson, L. F., Chalian, R., Foster, M. W., Gaughan, J. P., Coutifaris, C. and Sapienza, C. Inter- and intra-individual variation in allele-specific DNA methylation and gene expression in children conceived using assisted reproductive technology. *PLoS Genet* 2010;**6:**e1001033.

van Montfoort, A. P., Geraedts, J. P., Dumoulin, J. C., Stassen, A. P., Evers, J. L. and Ayoubi, T. A. Differential gene expression in cumulus cells as a prognostic indicator of embryo viability: a microarray analysis. *Mol Hum Reprod* 2008;**14:**157-168.

van Montfoort, A. P., Hanssen, L. L., de Sutter, P., Viville, S., Geraedts, J. P. and de Boer, P. Assisted reproduction treatment and epigenetic inheritance. *Human reproduction update* 2012;**18:**171-197.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A. and Speleman, F. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* 2002;**3:**research0034.0031-0034.0011.

Watkins, A. J., Platt, D., Papenbrock, T., Wilkins, A., Eckert, J. J., Kwong, W. Y., Osmond, C., Hanson, M. and Fleming, T. P. Mouse embryo culture induces changes in postnatal phenotype including raised systolic blood pressure. *Proc Natl Acad Sci U S A* 2007;**104:**5449-5454.

Watkins, A. J., Wilkins, A., Cunningham, C., Perry, V. H., Seet, M. J., Osmond, C., Eckert, J. J., Torrens, C., Cagampang, F. R., Cleal, J. et al. Low protein diet fed exclusively during mouse oocyte maturation leads to behavioural and cardiovascular abnormalities in offspring. *J Physiol* 2008;**586:**2231-2244.

Watkins, A. J., Lucas, E. S., Torrens, C., Cleal, J. K., Green, L., Osmond, C., Eckert, J. J., Gray, W. P., Hanson, M. A. and Fleming, T. P. Maternal low-protein diet during mouse pre-implantation development induces vascular dysfunction and altered renin-angiotensin-system homeostasis in the offspring. *The British journal of nutrition* 2010;**103:**1762-1770.

Wong, E. C., Hatakeyama, C., Robinson, W. P. and Ma, S. DNA methylation at H19/IGF2 ICR1 in the placenta of pregnancies conceived by in vitro fertilization and intracytoplasmic sperm injection. *Fertil Steril* 2011;**95:**2524-2526

Young, L. E., Fernandes, K., McEvoy, T. G., Butterwith, S. C., Gutierrez, C. G., Carolan, C., Broadbent, P. J., Robinson, J. J., Wilmut, I. and Sinclair, K. D. Epigenetic change in IGF2R is associated with fetal overgrowth after sheep embryo culture. *Nat Genet* 2001;**27:**153-154.

Chapter 6

Altered gene expression in human placentas after IVF/ICSI

Nelissen ECM, Dumoulin JCM, Busato F, Ponger L, Eijssen LMT, Evers JLH, Tost J, van Montfoort APA.

Human Reproduction. 2014;29(12):2821-31

Abstract

Study question: Is gene expression in placental tissue of IVF/ICSI patients altered when compared with a spontaneously conceived group, and are these alterations due to loss of imprinting (LOI) in the case of imprinted genes?

Summary answer: An altered imprinted gene expression of *H19* and Pleckstrin homology-like domain family A member 2 (*PHLDA2*), which was not due to LOI, was observed in human placentas after IVF/ICSI and several biological pathways were significantly overrepresented and mostly up-regulated.

What is known already: Genomic imprinting plays an important role in placental biology and in placental adaptive responses triggered by external stimuli. Changes in placental development and function can have dramatic effects on the fetus and its ability to cope with the intrauterine environment. An increased frequency of placenta-related problems as well as an adverse perinatal outcome is seen in IVF/ICSI derived pregnancies, but the role of placental epigenetic deregulation is not clear yet.

Study design and participants: In this prospective cohort study, a total of 115 IVF/ICSI and 138 control couples were included during pregnancy. After applying several exclusion criteria (i.e. preterm birth or stillbirth, no placental samples, pregnancy complications or birth defects), respectively, 81 and 105 placentas from IVF/ICSI and control pregnancies remained for analysis. Saliva samples were collected from both parents.

Methods: We quantitatively analysed the mRNA expression of several growth-related imprinted genes [*H19*, insulin-like growth factor 2 (*IGF2*), *PHLDA2*, cyclin-dependent kinase inhibitor 1C (*CDKN1C*), mesoderm-specific transcript homolog (*MEST*) isoform α and β by quantitative PCR] after standardization against three housekeeping genes [Succinate dehydrogenase A (*SDHA*), *YWHAZ* and TATA-binding protein (*TBP*)]. A quantitative allele-specific expression analysis of the differentially expressed imprinted genes was performed to investigate LOI, independent of the mechanism of imprinting. Furthermore, a microarray analysis was carried out (n = 10 in each group) to investigate the expression of non-imprinted genes as well.

Main results and the role of chance: Both *H19* and *PHLDA2* showed a significant change, respectively, a 1.3-fold ($P = 0.033$) and 1.5-fold ($P = 0.002$) increase in mRNA expression in the IVF/ICSI versus control group. However, we found no indication that there is an increased frequency of LOI in IVF/ICSI

placental samples. Genome-wide mRNA expression revealed 13 significantly overrepresented biological pathways involved in metabolism, immune response, transmembrane signalling and cell cycle control, which were mostly up-regulated in the IVF/ICSI placental samples.

Limitations, reasons for caution: Only a subset of samples was found to be fully informative, which unavoidably led to lower sample numbers for our LOI analysis. Our study cannot distinguish whether the reported differences in the IVF/ICSI group are exclusively attributable to the IVF/ICSI technique itself or to the underlying subfertility of the patients.

Wider implications of the findings: Whether these placental adaptations observed in pregnancies conceived by IVF/ICSI might be connected to an adverse perinatal outcome after IVF remains unknown. However, it is possible that these differences affect fetal development and long-term patterns of gene expression, as well as maternal gestational physiology.

Study funding/competing interest(s): Partly funded by an unrestricted research grant by Organon BV (now MSD BV) and GROW School for Oncology and Developmental Biology without any role in study design, data collection and analysis or preparation of the manuscript. No conflict of interests to declare.

Trial registration number: Dutch Trial Registry (NTR) number 1298.

Introduction

The placenta is a highly specialised and adaptive organ, which supports fetal growth and development. Changes in placental development and function can have dramatic effects on the fetus and its ability to cope with the intrauterine environment. Placental adaptive responses that are triggered by in- or extrinsic factors may have long-lasting consequences, as intrauterine growth and size at birth are critical determinants for the successful adaptation to post-natal life. The origin of chronic diseases in later life, such as hypertension, type 2 diabetes, coronary heart disease and stroke, can be traced back to intrauterine growth, according to the developmental origins of health and disease (DOHaD) concept (Barker *et al.*, 2009).

In human IVF/ICSI offspring, a lower birthweight (Pandey *et al.*, 2012) as well as characteristics of metabolic syndrome, such as vascular dysfunction, increased systolic blood pressure, fasting glucose level and skin fold thickness (Ceelen *et al.*, 2007; Ceelen *et al.*, 2008; Sakka *et al.*, 2010; Scherrer *et al.*, 2012), have been reported. An important role for the placenta herein is plausible as an increased frequency of placenta-related problems is observed in IVF/ICSI derived pregnancies, such as an increased risk of hypertensive disorders of pregnancy, placenta praevia, abruption and third trimester vaginal bleeding (Pandey *et al.*, 2012). Recently, a higher placental weight/birthweight ratio among pregnancies conceived by assisted reproduction technologies (ART) compared with spontaneous pregnancies was found, which persisted after adjustment for length of gestation, offspring birthweight, parity, fetal sex, maternal age, pre-eclampsia and diabetes (Haavaldsen *et al.*, 2012).

Genomic imprinting plays an important role in placental biology and in the placental adaptive responses (Sandovici *et al.*, 2012). Imprinted genes, which are expressed in a parent-of-origin-dependent way, are abundantly expressed in the placenta and are indispensable for proper placental morphology and function (Kawahara *et al.*, 2009; Nelissen *et al.*, 2011). Transcription of either the paternal or the maternal gene is regulated by epigenetic marks, such as DNA methylation, histone modifications or non-coding RNAs (Nelissen *et al.*, 2011). Around conception, genome-wide epigenetic reprogramming takes place but imprinted genes are protected to preserve the parental imprints in the developing embryo (Reik *et al.*, 2001; van Montfoort *et al.*, 2012). This period of epigenetic reprogramming is known to be sensitive to epigenetic

disturbances (Fraga *et al.*, 2005; Jirtle *et al.*, 2007), which might potentially make ART treatment a risk.

An important question is whether IVF/ICSI induces epigenetic deregulation of imprinted genes in placental tissue. Indeed, animal models have shown that for instance preimplantation embryo culture can affect methylation and expression of imprinted genes (Mann *et al.*, 2004; Fauque *et al.*, 2010; Market-Velker *et al.*, 2010). Moreover, animal studies suggest that placental tissues are more sensitive to preimplantation epigenetic disturbance of imprinted genes than embryonic tissues (Mann *et al.*, 2004; Rivera *et al.*, 2008). This can lead to abnormal placental development and function with possible consequences for the developing fetus.

In humans, several studies performed in the oocyte, pre-implantation embryo, peripheral blood, umbilical cord blood, amniotic membrane, cord and buccal smears have shown conflicting data about epigenetic deregulation (Geuns *et al.*, 2003; Sato *et al.*, 2007; Tierling *et al.*, 2010; Feng *et al.*, 2011; Hiura *et al.*, 2012; Oliver *et al.*, 2012; Puumala *et al.*, 2012; Huntriss *et al.*, 2013). However, these studies, although of great value, cannot easily be compared with each other and transferred to placenta biology because genomic imprinting can be tissue-specific, especially in extra-embryonic tissues (Tabano *et al.*, 2010; Prickett *et al.*, 2012).

Only a few studies have focused on the epigenetic deregulation after IVF/ICSI in human placenta. DNA methylation differences of imprinted genes and the *H19/IGF2* imprinting control region (*H19 ICR1*) have been reported (Katari *et al.*, 2009; Turan *et al.*, 2010; Rancourt *et al.*, 2012). In a previous study, we identified aberrant DNA methylation at some differentially methylated regions (DMRs), which in some cases did *(H19)* and some cases did not *(MEST)* lead to aberrant gene expression (Nelissen *et al.*, 2013). Therefore, it is important to not only investigate DNA methylation, but also to focus on placental gene expression.

Furthermore, it is currently unknown whether the reported changes in imprinted gene-expression are the result of loss of imprinting (LOI), i.e. expression from the normally silenced allele. There is evidence that histone methylation and non-coding RNAs rather than DNA methylation might be important for imprinting maintenance in the mouse placenta (Lewis *et al.*, 2004; Wagschal *et al.*, 2006; Pandey *et al.*, 2008; Wagschal *et al.*, 2008; Redrup *et al.*, 2009). Therefore, we investigated LOI, independently of the mechanism of imprinting, using an allele-specific expression analysis.

Besides imprinted genes, also non-imprinted genes are important for placental development (Nelissen *et al.*, 2011). Katari *et al.* found that *in vitro* conception-associated DNA methylation differences are associated with gene expression differences at both imprinted and non-imprinted genes (Katari *et al.*, 2009). Furthermore, it is known that gene expression changes in placental transporter systems can be induced by intrinsic (i.e. genetic and developmental) and extrinsic (i.e. environmental) factors (Sandovici *et al.*, 2012).

Our hypothesis is that epigenetic aberrations occur in placental tissue of IVF/ICSI patients compared with placental tissue of spontaneous pregnancies. Therefore, we carried out a microarray analysis to investigate genome-wide which pathways are deregulated in IVF/ICSI placentas. Regarding the importance of imprinted genes in placental function, special attention was given to several growth-related imprinted genes (*H19, IGF2, PHLDA2, CDKN1C, MEST* isoform *a* and β) by analysing the expression in a larger number of samples using quantitative real-time PCR (qPCR) after standardisation against three housekeeping genes (*SDHA, YWHAZ* and *TBP*). Finally, a quantitative allele-specific expression analysis of the differentially expressed imprinted genes was performed to investigate LOI, independent of the mechanism of imprinting.

Material and Methods

Study population and sample collection

In this prospective cohort study, IVF/ICSI patients were recruited at the IVF centre of the Maastricht University Medical Centre (MUMC), The Netherlands, after an ultrasound examination of a viable singleton pregnancy at 7 weeks' gestation in the period between May 2008 and October 2009. Only pregnancies resulting from fresh embryo transfers after standard IVF or ICSI procedures were included. Excluded were patients who had performed PGD or required donor gametes. IVF and ICSI treatments were performed as described previously (Dumoulin *et al.*, 2010), with the exception that in this study only Vitrolife G1.5 medium (Göteborg, Sweden) was used.

Non-IVF control pregnant couples were recruited during their pregnancy in the period between April 2008 and July 2009 at the obstetric outpatients' clinic in the MUMC (52%) and at cooperating midwife practices

(48%), and were defined as having conceived spontaneously without the use of any kind of hormones, other medication or ART. Also in this group only singleton pregnancies were included.

In both groups, saliva samples were collected from both parents for genomic DNA extraction using the Oragene® DNA Self-Collection kit (DNA Genotek Inc., Ottawa, Canada). Participants were not allowed to eat, drink, smoke or chew gum for half an hour before producing the saliva sample (2 mL). The couples were asked to fill in a short questionnaire about their height and weight before pregnancy and the smoking and drinking habits of the woman before and during the pregnancy.

Placental tissue was collected within 30 minutes after delivery of the placenta by the gynaecologist, nurse or midwife according to a standardized protocol. The biopsies (~5 mm^3) were taken from the fetal side near the umbilical cord insertion point, rinsed extensively in cold phosphate-buffered saline to remove blood and stored in RNAlater® (Applied Biosystems/Ambion, USA). The samples were sent to the department of Obstetrics & Gynaecology at the Maastricht University Medical Centre together with a copy of the delivery report. After receiving the samples, the amnion and chorionic membranes were removed (which also served as an extra verification that the biopsies were taken from the fetal side) and the villi were stored until further use at -20°C according to the guidelines provided by the RNAlater manufacturer. Samples from deliveries before 37 weeks' gestation were excluded from analysis. Also samples from patients with pregnancy complications, such as gestational diabetes, hypertension and pre-eclampsia, or from children with birth defects, were excluded.

This study was approved by the Ethical Review Board of the Maastricht University Medical Centre and all participants gave written informed consent.

RNA isolation from placental tissues

Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions with some minor adaptations. After adding 800 µL Trizol with 10% β-mercaptho-ethanol, the samples were ground using a pestle and a mini bead beater (Biospec, USA). RNA was precipitated with isopropyl alcohol at room temperature for 2 h and the RNA pellet was washed three times with 70% ethanol. Total RNA was resuspended in 20 µL RNAse free water and stored at -80°C. RNA quantity and quality were

determined using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, USA) and RNA integrity was measured using a Bioanalyzer 2100 (Agilent technologies, Palo Alto, USA).

cDNA synthesis

After a DNAse I treatment (Invitrogen, Carlsbad, USA), total RNA was converted to cDNA using the High Capacity cDNA Reverse transcription kit (Applied Biosystems, Foster City, USA) according to the manufacturer's instructions.

Quantitative PCR

Each cDNA sample was diluted 500 fold. All assays were run in triplicate in a reaction mixture containing 1 µL of the diluted sample, 5 µL reaction buffer of mesa green qPCR MasterMix plus for SYBR assay (Eurogentec, San Diego, USA), 1 µL of each primer ([1µM]), 1.9 µL nuclease-free water and 0.1 µL Uracil-N-Glycosylase (UNG, Eurogentec). The investigated genes of interest were *H19, IGF2, PHLDA2, CDKN1C, MEST isoform a* and β, which are all growth-related genes. The primers are listed in Supplementary data, Table SI and were chosen to span an exon-exon barrier wherever possible. qRT-PCR amplification was performed using an Applied Biosystems Prism 7900 sequence detection system with the following thermal cycler conditions: 2 min at 50°C for UNG treatment, 10 min at 95°C to activate Taq polymerase and 40 cycles of denaturation at 95°C for 15 s and annealing and elongation at 60°C for 1 min. A dissociation curve analysis was performed to verify the absence of genomic DNA contamination and to control whether the reaction was specific for one PCR product.

The expression of each gene was related to the expression of a combination of reference genes as described previously (Nelissen *et al.*, 2013). The mean of the Ct values of the reference genes *SDHA, YWHAZ* and *TBP* was used as a normalisation factor and subtracted from the Ct values of the genes of interest to obtain normalized Ct values (ΔCt). The fold change in expression of each gene of interest was analyzed using the ΔΔCt method (Livak *et al.*, 2001).

Microarray analysis

Two hundred nanogram of total RNA was amplified using the Ambion WT Expression Kit (Life technologies, Carlsbad, CA, USA). The Affymetrix WT Terminal Labeling Kit was used for fragmentation and labeling (Affymetrix, Santa

Clara, USA). Hybridization was performed using 5 micrograms of biotinylated target, which was incubated with the GeneChip® Human Gene 1.0 ST array (Affymetrix) at 45^{0}C for 16–18 h, covering 36,079 transcripts. After hybridization, nonspecifically bound material was removed by washing and specifically bound target was detected using the GeneChip Hybridization, Wash and Stain kit, and the GeneChip Fluidics Station 450 (Affymetrix). The arrays were scanned using the GeneChip Scanner 3000 7G (Affymetrix) and raw data were extracted from the scanned images. The samples were processed in two separate batches of both IVF/ICSI and control samples (4 IVF/ICSI + 2 controls and 6 IVF/ICSI + 8 controls).

Genomic DNA extraction from placenta and saliva

DNA from placental tissue was extracted using the Gentra PureGene tissue kit (Qiagen, Venlo, the Netherlands) according to the manufacturer's guidelines. The AutoGenFlex STAR (Autogen, Holliston, USA) was used for fully automated extraction of DNA from saliva samples according to the manufacturer's instructions.

Allele-specific expression analysis

Single Nucleotide Polymorphisms (SNPs) were used to distinguish between the parental alleles of *H19* and *PHLDA2*. Genotyping of SNPs in genomic DNA of placental tissue and saliva samples from both parents, was done by PCR and sequencing according to the Sanger (chain-termination) method. The PCR primers, which contained an M13 tail for sequencing, and the analysed SNPs are listed in Supplementary data, Table SII. PCR amplification of *H19* was performed using an initial denaturation step at 94°C for 3 minutes followed by 35 cycles of denaturation at 94°C for 30 s, annealing for 40 s at 61°C and elongation at 72°C for 1 min. The program ended with a final extension step at 72°C for 10 min. For *PHLDA2*, the thermal cycling conditions were the same except for the annealing temperature of 55°C, an elongation time of 3 min and 45 cycles of amplification. DNA sequencing of the PCR products after amplification was performed by using the Big Dye terminator cycle sequencing ready reaction mix in combination with M13 primers and an ABI 3730 DNA Analyser (Applied Biosystems).

In placental samples that were informative (i.e. the parental alleles could be distinguished), the parent-of-origin-specific expression of imprinted

genes was determined quantitatively by pyrosequencing of cDNA. All allele-specific expression analyses were performed in triplicate. Regions of interest were amplified using 20 ng of cDNA, and 5 pmol of forward and reverse primer, one of them being biotinylated. Sequences for oligonucleotides for PCR amplification and pyrosequencing are given in Supplementary data, Table SIII. Reaction conditions were 10X HIFI Platinum Taq buffer supplemented with 25 mM MgCl2, 10 mM dNTPs and HIFI Platinum Taq (Life Technologies) in a 25 µL volume. The PCR program consisted of a denaturing step of 4 min at 95°C followed by 50 cycles of 30 s at 95°C, 30 s at the respective annealing temperature and 15 s at 72°C, with a final extension of 4 min at 72°C. Ten µL of PCR product was rendered single-stranded as previously described (Tost *et al.*, 2007) and 4 pmol of the respective sequencing primer was used for analysis. Quantitative allelic expression analysis was carried out on a PSQ 96MD system with the PyroGold Q96 Reagent Kit (Qiagen) and results were analyzed using the PyroMark MD software (V1.0., Qiagen).

Statistical analysis

Statistical analysis was performed using SPSS software for windows version 20.0 (Statistical Package for Social Sciences, USA). To test differences, Student's t-test was used for continuous variables and the Chi-Square test was used for binary variables. The difference in relative gene expression between both groups, as analyzed by qPCR, was tested with the non-parametric Mann-Whitney U test. $P < 0.05$ was considered significant.

Microarray data analysis was performed using the R statistical software (http://cran.r-project.org/). A variety of established array specific quality control, visualization, normalization and statistical methods were combined into one workflow at the BiGCaT department (Eijssen *et al.*, 2013). Additionally, we corrected for batch as a possible covariate. Linear modelling using the limma package was conducted to compute the genes that were significantly changed between experimental groups, as defined by a p-value smaller than 0.05. These genes were mapped to biological pathways using PathVisio (van Iersel *et al.*, 2008; Kelder *et al.*, 2011). Using the statistics function in PathVisio, an ordered list of Z-score ranked pathways was generated based on the overrepresentation of member genes that were significantly changed between experimental groups. All pathways with a Z-score higher than 1.96 were included in the biological interpretation, which is comparable to a significance level of 0.05.

Outlier detection for allele-specific expression was performed by using tests available in the Outliers and the Extreme values packages developed for the R statistical software (http://cran.r-project.org/). Several methods of outlier detection were performed since they all have advantages and disadvantages. The Grubb test (Grubbs, 1969) which is based on the assumption of normality and the Dixon test (Dixon, 1950) which is distribution-free have been used to detect individual outliers. Identified outliers were removed from the data and the test was iterated until no further outliers were detected. However, the iteration process can be prematurely stopped if one outlier masks a second outlier (masking effect). The generalized Extreme Studentized Deviate (gESD) test (Rosner, 1983) and the Loo tests (van der Loo, 2010) are designed to detect one or more outliers simultaneously among data that follow an approximately normal distribution. Samples exhibiting a modified Z-score (Iglewicz *et al.*, 1993) with an absolute value greater than 3.5 have been labelled as potential outliers, as suggested by the authors.

Results

Sample collection and group comparison

A total of 115 IVF/ICSI and 138 control couples were included in the study after having signed an informed consent (Figure I). In the IVF/ICSI group 34 couples were excluded because either no biopsies were taken (n=29), samples were collected after a stillbirth (n=1) or from patients with pregnancy complications (n=2, hypertension and HELLP syndrome) or a preterm delivery (<37 weeks, n=2), resulting in 81 placenta samples for analysis. The control group contained only term deliveries and no patients with pregnancy complications. However, in 32 cases no biopsies were taken and in one case there was a birth defect (trisomy 18), which resulted in 105 placentas for analysis. In Table I parental and neonatal characteristics from both groups are shown. With the exception of the age of the parents, all characteristics are similar. During pregnancy, there were two women (one in either group) who used alcohol sporadically and two women in the IVF group who used recreational drugs on one occasion (data not shown).

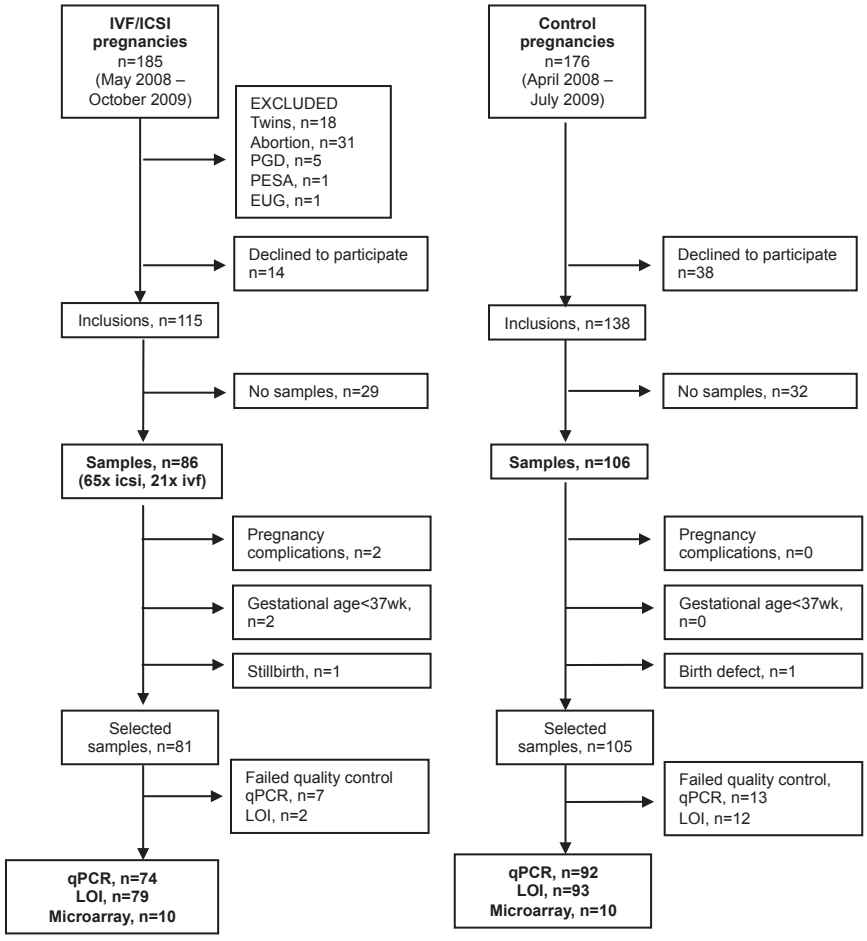


Figure I Flowchart

Microarray analysis

Ten IVF/ICSI (6 males, 4 females) and 10 control (4 males, 6 females) samples were randomly selected for microarray analysis. All indicators of sample quality, hybridization quality, signal comparability and array correlation met the quality control criteria, so that no array needed to be excluded from the dataset. We corrected for batch as possible covariate. The number of significantly differentially expressed genes (DEGs) between IVF/ICSI and control placentas was calculated. There were 839 (47.5%) upregulated and 927 (52.5%) downregulated DEGs, which was comparable. Most DEGs showed a

Table I Neonatal and parental characteristics

	Control (n=105)	IVF/ICSI (n=81)	*P*-value
Birthweight (g)	3501 ± 445	3460 ± 576	NS
Gestational age (weeks)	39.7 ± 0.9	40.0 ± 1.3	NS
Z-score	-0.002 ± 0.95	-0.12 ± 1.11	NS
Boys	54 (51.4)	42 (51.9)	NS
Mother			
Age (years)	31.1 ± 4.6	33.9 ± 4.1	<0.001
BMI	24.1 ± 4.4	23.9 ± 3.3	NS
Primipara	55 (52.4)	54 (66.7)	NS
Smoking during pregnancy	11 (10.5)	7 (8.6)	NS
0-9 cigarettes/day	9 (8.6)	4 (4.9)	
≥10 cigarettes/day	2 (1.9)	3 (3.7)	
Father			
Age (years)	33.5 ± 5.1	36.3 ± 5.8	0.002
BMI	25.2 ± 3.7	25.8 ± 3.6	NS

Data are presented as numbers (%) or mean ± SD. NS; not significant. Student's *t*-test.

fold change between -1.5 and +1.5. The number of DEGs >1.5 fold upregulated or <1.5 fold downregulated was, respectively, 14 and 6.

Pathway analysis of the DEGs in placental tissue from IVF/ICSI and spontaneous pregnancies revealed 13 significantly overrepresented biological pathways, for instance the miRNA Regulation of DNA damage response, cholesterol biosynthesis, one-carbon metabolism and electron transport chain pathway. All 13 pathways and their calculated Z-scores are summarized in Table II.

Gene expression analysis

The investigated genes of interest were *H19, IGF2, PHLDA2, CDKN1C, MEST isoform α* and β, which are all growth-related genes. After quality control of the qPCR results as described in the methods section, we excluded another 7 samples in the IVF/ICSI group and 13 in the control group (Figure I). The relative expression levels of *H19, IGF2, PHLDA2, CDKN1C, MEST* isoform *α* and β were obtained in 166 placental samples (92 in the control group and 74 in the IVF/ICSI group) after standardisation against the housekeeping genes *SDHA, YWHAZ* and *TBP*. Both *H19* and *PHLDA2* showed a significant increase in expression in the IVF/ICSI versus control group, respectively 1.3-fold ($P = 0.033$) and 1.5-fold ($P = 0.002$) (Figure II). Additionally, we performed a linear regression analysis per imprinted gene with group (IVF/control), maternal and paternal age as possible

covariates. Only group was significantly associated with gene expression of *H19* and *PHLDA2*, while parental age was not.

Table II Pathways significantly overrepresented in the differentially expressed genes (DEGs) list

Pathway	Positive (r)	Measured (n)	Total	%	Z-score
RB in Cancer	25	98	104	25.51	6.58
Gastric cancer network I	8	26	29	30.77	4.37
One Carbon Metabolism	6	24	51	25.00	3.14
Heme Biosynthesis	3	8	28	37.50	3.13
DNA replication	8	40	50	20.00	2.88
Electron Transport Chain	15	96	118	15.63	2.87
Oxidative phosphorylation	10	56	69	17.86	2.81
Type III interferon signalling	3	10	11	30.00	2.61
Statin Pathway	6	29	46	20.69	2.59
Cholesterol Biosynthesis	4	16	33	25.00	2.56
miRNA Regulation of DNA Damage Response	12	80	106	15.00	2.41
Fluoropyrimidine Activity	6	31	44	19.35	2.40
TFs Regulate miRNAs related to cardiac hypertrophy	3	12	16	25.00	2.22

miRNA; microRNA; RB; retinoblastoma protein; TF; transcription factors.

Allele-specific expression analysis

Next, we investigated whether the significantly increased expression of *H19* and *PHLDA2* in the IVF/ICSI placental samples was the result of LOI leading to expression of the silent paternal allele. Pyrosequencing has a detection limit of 5% (Ogino *et al.*, 2005). Therefore, we considered any result below 5% as technical noise. For SNP rs10840159 the observed background signal was higher and the threshold was set to 10%.

For the investigation of the imprinting status of *PHLDA2,* genomic DNA from 172 placental samples was tested for heterozygosity at SNP rs1056819 (A/G); 46 samples were found to be informative. Saliva samples from the corresponding 46 parent-couples were tested of which 21 appeared informative (9 control and 12 IVF/ICSI). Quantitative allele-specific expression analysis of the cDNA from 21 placental samples revealed LOI in 11 placental samples (52.4%), varying from 6% to 13.6% paternal expression (Figure IIIA and IIIC). The mean rate of paternal expression in the control group (n=5) was 10.1% compared with

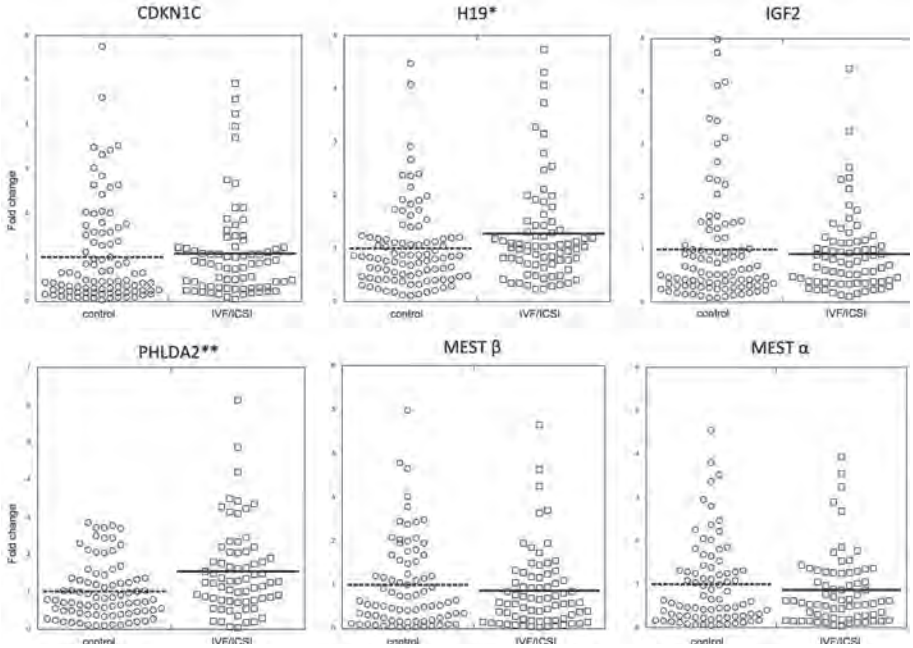
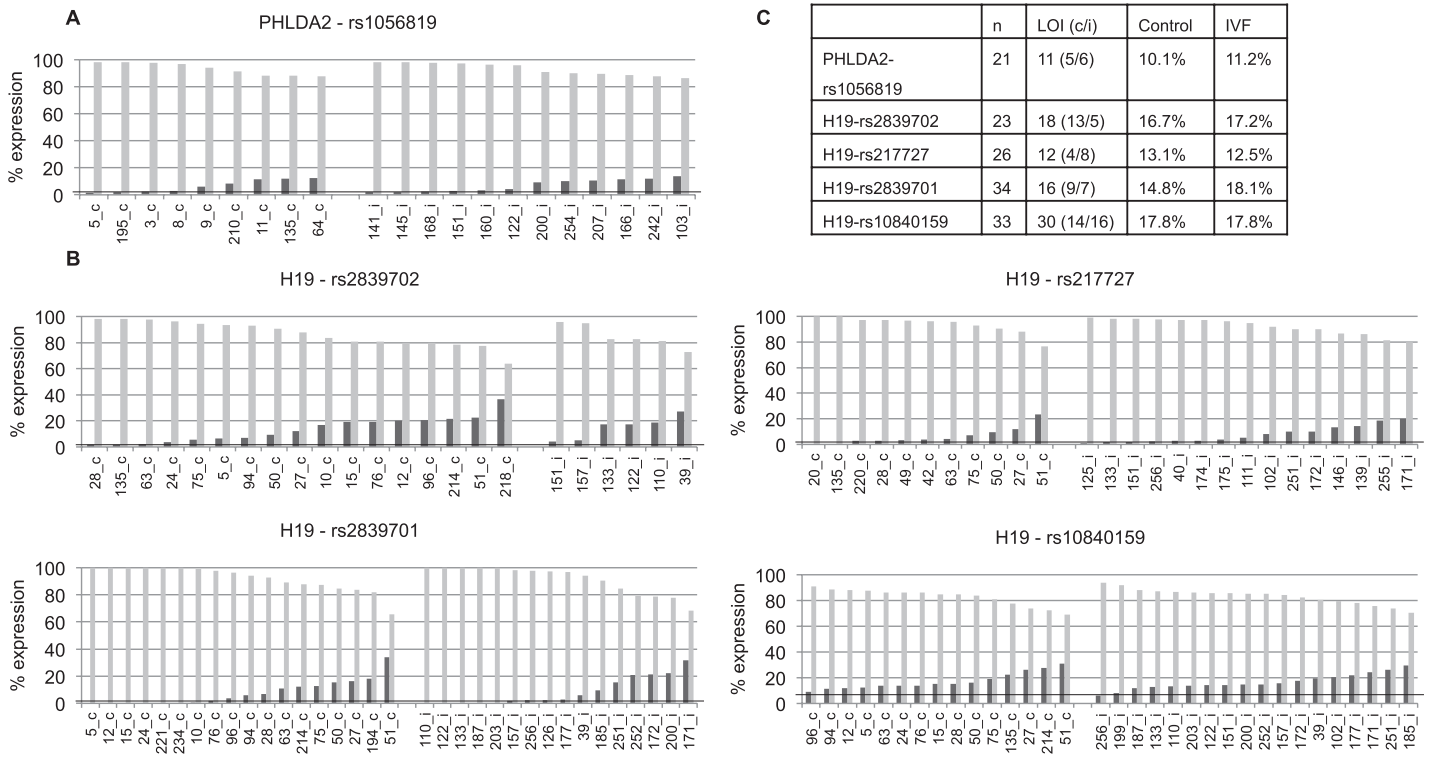


Figure II Relative gene expression of imprinted genes in placental tissue from spontaneous (control; n=92) and IVF/ICSI pregnancies (n=74). Control group is set at one. * $P < 0.05$, ** $P < 0.01$.

11.2% in the IVF group (n=6), which was not significant (Mann-Whitney *U*-test). According to the LOO I outlier detection method, two samples (242 and 103) displayed abnormal paternal expression (Supplementary data, Table SIV). Both samples belong to the IVF group.



	n	LOI (c/i)	Control	IVF
PHLDA2-rs1056819	21	11 (5/6)	10.1%	11.2%
H19-rs2839702	23	18 (13/5)	16.7%	17.2%
H19-rs217727	26	12 (4/8)	13.1%	12.5%
H19-rs2839701	34	16 (9/7)	14.8%	18.1%
H19-rs10840159	33	30 (14/16)	17.8%	17.8%

Figure III Allele-specific expression of *PHLDA2* and *H19* in term placental samples

The x-axis indicates the placental samples; percentage expression from each allele is indicated on the y-axis. Light gray bar indicates percentage of maternal expression; dark gray bar represents percentage of paternal-specific expression. PHLDA2 (**A**) was investigated at the SNP (rs1056819) and H19 (**B**) at four SNPs (rs2839701, rs2839702, rs10840159 and rs217727). Black line represents the detection limit of 10% at SNP H19-rs10840159 and 5% at the other SNPs. C, controls; i, IVF/ICSI. In the table (C), the total number of informative samples, samples with LOI and the mean percentages of paternal expression are shown (not significant, Mann-Whitney U test).

For the *H19* LOI analysis, the placental genomic DNA was tested for heterozygosity at four SNPs (C/G,rs2839701; G/T,rs2839702; A/G,rs10840159; C/T,rs217727), 90 samples were found to be informative for at least one SNP. Saliva samples from 180 parents were tested for heterozygosity and 52 sample-couples were informative. Quantitative allele-specific expression analysis revealed differences among the investigated SNPs (Figure IIIB and IIIC). LOI was observed in 18/23 (78.3%) samples at SNP rs2839702 (5.1% to 36.4% paternal expression) and in 30/33 (90.9%) samples at SNP rs10840159 (11.5% to 30.9% paternal expression). LOI was less common at SNP rs217727 (12/26, 46.2%) and rs2839701 (16/34, 47.1%), with paternal expression rates ranging from 5.1% to 23.5% and 5.8% to 34.2%, respectively. Although the mean rate of paternal expression appeared slightly higher in the IVF group at three of the five investigated SNPs, the difference was not significant (Figure IIIC). Supplementary data, Table SIV displays the summary of the outlier search using univariate methods. Most tests defined sample 185 (IVF) and 214 (control) as outlying samples. It is interesting to note, that sample 185 was defined as an outlier at all informative SNPs (2/2) and 214 at two out of three SNPs (Figure IIIB). In the 3rd SNP (rs2839702) of sample 214, the paternal expression was also among the highest. Sample 218 (control) was only informative at one SNP, but was found in several outlier tests. The characteristics of the LOI outliers were also compared with the characteristics of the non-outliers (Table III), which revealed no differences. Altogether, there is no evidence that there is more LOI in IVF placental samples.

Table III Characteristics of loss of imprinting outliers in comparison with the non-outliers

	Outliers	Non-outliers	*P*-value
Birthweight (g)	3296 ± 442	3507 ± 517	NS
Range	2650 - 3860	2360 - 5185	
Gestational age (weeks)	39.5 ± 0.9	39.8 ± 1.1	NS
Range	38.1 - 40.9	37.0 - 41.9	
Z-score	-0.40 ± 0.81	-0.01 ± 1.04	NS
Range	-1.440 - 0.598	-2.576 - 2.576	
Mother			
Age (years)	34.3 ± 6.1	32.0 ± 5.2	NS
Range	22 - 39	21 - 42	
BMI	22.6 ± 2.3	24.0 ± 4.0	NS
Range	20 - 26	18 - 43	
Father			
Age (years)	34.0 ± 5.5	34.6 ± 5.6	NS
Range	25 - 38	20 - 58	
BMI	24.7 ± 2.1	25.5 ± 3.6	NS
Range	22 - 29	18 - 39	

Data are presented as mean ± SD. NS; not significant. Student's *t*-test.

Discussion

Genome-wide mRNA expression analysis in placentas from IVF/ICSI derived pregnancies and spontaneously conceived pregnancies revealed 13 significantly overrepresented biological pathways. These pathways can be roughly clustered according to their function into metabolism, immune response, transmembrane signalling and cell cycle control as indicated by the cancer pathways. In the RB in Cancer pathway, the *BRCA1*, *CDK1* and *CCNA2* genes were all upregulated in the placental samples from the IVF/ICSI group. These genes play a role in maintaining genomic stability and cell cycle control. Also in the Gastric Cancer Network, DNA replication and miRNA Regulation of DNA damage response pathway, all differentially expressed genes were upregulated and again important for cell cycle control. This could imply that ART manipulations or the parental subfertility lead to enhanced cell cycle control mechanisms, although the cell cycle pathway itself was not significantly upregulated according to the pathvisio analysis (10 out of the 100 genes in this pathway were significantly altered between IVF and control placentas, z-score=0.81). Other affected

pathways play a role in metabolism. In the cholesterol biosynthesis and heme biosynthesis pathways, all differentially expressed genes were upregulated in placental samples from the IVF/ICSI group. Cholesterol is of vital importance for fetal development as a key constituent of cell membranes, precursor of steroid hormones and metabolic regulators. The placenta regulates maternal-fetal cholesterol transport and a disturbed regulation can lead to atherogenic programming in the offspring (Palinski, 2009). Hemes are involved in oxygen transport, detoxification and signal transduction processes (Ryter *et al.*, 2000). Most of the DEGs in the electron transport chain pathway (drives ATP synthesis) and in the one-carbon metabolism pathway are also upregulated: the latter is important for the production of purines and pyrimidines (required for synthesis of DNA and RNA) as well as S-adenosylmethionine (substrate for e.g. DNA methylation). Furthermore, the statin pathway and oxidative phosphorylation pathway have DEGs that are both up- and downregulated. According to all these altered metabolism pathways, an altered metabolic activity in placentas of IVF/ICSI pregnancies might be an adaptive mechanism to ART-imposed stress or a subfertility-induced deregulation, which potentially leads to a poorer outcome in ART pregnancies. Our results are however still too preliminary to elucidate these questions.

A small study by Zhang *et al.* (2010) investigated global gene expression in three term placentas from IVF pregnancies compared with three placentas from spontaneous pregnancies. They found 18 DEGs and classified them according to their role in biological processes in immune response, transmembrane transport, metabolism, oxidative stress, cell differentiation and other processes. These pathways are strikingly comparable to those found in our study. Furthermore, an extensive study on the placental transcriptome after ART in mice also revealed comparable results. IVF techniques in mice triggered the induction of genes involved in cellular proliferation and cell cycle pathways and an alteration of gene expression involved in apoptotic pathways (Fauque *et al.*, 2010). They also observed a decrease in expression of genes involved in the angiogenic signalling and immune response.

Subsequently, we analysed the gene expression of several placental imprinted genes (*H19, IGF2, PHLDA2, CDKN1C, MEST* isoform *α* and β) in a larger sample size (n=166) by qPCR. We chose to investigate these particular genes as they are all growth-related genes and IVF children are known to have a significantly lower birthweight (Pandey *et al.*, 2012). *MEST* and *IGF2* are

paternally expressed genes, which favour placental and fetal development. In contrast, *H19, PHLDA2* and *CDKN1C* are maternally expressed and are growth inhibitory genes (Tycko *et al.*, 2002; Tycko, 2006). Both *H19* (a non-coding RNA) and *PHLDA2* (the pleckstrin homology-like domain, family A, member 2 gene) showed a significant increase, respectively of 1.3-fold and 1.5-fold, in expression in the IVF/ICSI group. *PHLDA2* expression was also upregulated according to our microarray analysis (fold change 1.2; data not shown). Not entirely unexpected, two growth inhibitory genes were upregulated in placental samples from IVF/ICSI derived pregnancies. In human studies, an increased (1.3 to 2.8-fold) *PHLDA2* expression was found in the placenta of intrauterine growth restricted (IUGR) babies (McMinn *et al.*, 2006; Diplas *et al.*, 2009; Kumar *et al.*, 2012), and an association between elevated placental *PHLDA2* expression levels and low birthweight was described (Apostolidou *et al.*, 2007). However, it is important to note that we did not find any significant differences in birthweight between the groups. Additionally, Turan *et al.* found significantly lower transcripts of both *IGF2* and *H19* in placentas from the *in vitro* group (Turan *et al.*, 2010).

Several studies on epigenetic deregulation in placentas after IVF/ICSI treatments focussed on DNA methylation as a possible cause for altered gene expression of imprinted genes. In mice, a loss of *H19* methylation and/or an increase in the biallelic expression of *H19* was found specifically in the placenta after ovulation induction (Fortier *et al.*, 2008) or embryo culture (Mann *et al.*, 2004; Rivera *et al.*, 2008). Katari et al. investigated genes with methylation differences in placental samples of in vitro or in vivo conceived children and found that *MEST* and *SERPINF1* showed a higher average transcript level in the *in vitro* group (Katari *et al.*, 2009). In our previous study, we found a reduced DNA methylation level at the *H19* and *MEST* DMRs, and an increased *H19* expression in placentas from pregnancies conceived by IVF/ICSI (Nelissen *et al.*, 2013). A correlation between *H19* hypomethylation and increased expression was also reported by Rancourt *et al.* (2012) in placentas from pregnancies achieved with ovulation induction and/or IVF.

Next, we investigated whether the significantly increased expression of *H19* and *PHLDA2* in the IVF/ICSI placental samples was the result of LOI leading to expression of the silent paternal allele. To the best of our knowledge, LOI has never been investigated in human IVF/ICSI placental samples before. LOI was common at two SNPs of *H19* (90.9% and 78.3% of placental samples showing LOI) and less common at the other SNPs of *H19* and *PHLDA2* (46.2-52.4%).

Although the mean LOI appeared slightly higher in the IVF group at three of the five investigated SNPs, there was no difference in mean LOI between the control group and the IVF group (non-parametric test). Our study shows that LOI is common in placental samples (whether or not conceived via IVF) which is in agreement with two previous reports (Lambertini *et al.*, 2008; Rancourt *et al.*, 2013). Lambertini *et al*. found a wide range of LOI in human placenta varying from 0 to 96% (with 25% of the informative polymorphisms showing LOI>3%), depending on the gene (i.e. *H19, IGF2, MEST*) and additionally the type of pregnancy complications (i.e. pre-eclampsia or IUGR). Rancourt *et al.* (2013) found LOI of *H19* in placentas of phenotypically healthy individuals, while complete maintenance of *IGF2* mono-allelelic expression was observed in all these placental samples. Although there was a slightly higher mean LOI in the IVF/ICSI placental samples, there are currently no indications that the significantly increased expression of *H19* and *PHLDA2* in the IVF/ICSI placental samples is related to a higher expression of the silent paternal allele. Possibly, the smaller sample size in our LOI analysis, being dependant on the number of informative heterozygous SNPs, could have influenced the significance of the results. Certainly, this needs further investigation. The outlier analysis showed no difference between the IVF/ICSI group and the control group.

How the differences in gene expression are regulated, what exactly causes these differences and what the clinical relevance is, remains so far unknown. The molecular mechanisms that underlie placental adaptations *in vivo* are mostly unknown and are a challenge to study given the complexity of the maternal-placental-fetal interactions. Placental adaptations may either affect fetal development directly, or indirectly by changing the maternal gestational physiology, for instance through an effect on maternal appetite or maternal disease such as gestational diabetes and pre-eclampsia (Sandovici *et al.*, 2012). Even though we analysed the fetal side of the placenta that is derived from trophoblast cells which have surrounded the *in vitro* exposed embryonic cells, our study cannot distinguish whether the reported gene expression differences in the IVF/ICSI versus control group are exclusively attributable to the IVF/ICSI technique itself or to the underlying subfertility of the patients.

A shortcoming of our study is that only a subset of samples was found to be fully informative for LOI analysis. Although comparable to another study (Apostolidou *et al.*, 2007), this implies less samples for LOI analysis and therefore less conclusive power. Another issue is that there was no difference in

birthweight between IVF/ICSI children and spontaneously conceived children, and that placental related problems, i.e. pre-eclampsia and hypertension, were excluded from our study. This could possibly explain the subtle differences between groups and perhaps we have missed more severe differences with this selection.

In conclusion, an altered gene expression occurs in placental tissue of IVF/ICSI patients when compared with placental tissue of spontaneous pregnancies in both non-imprinted and imprinted genes: the latter was not due to LOI. Furthermore, several biological pathways were significantly overrepresented and mostly upregulated in the IVF/ICSI placental samples. Whether these are adaptations or deregulations, and whether these are connected to the adverse perinatal outcome after IVF remains unknown. However, it is imaginable that these differences have the potential to affect fetal development either directly or indirectly by changing the maternal gestational physiology. Altogether, these changes may lead to long-term patterns of fetal gene expression that might be associated with an increased risk for (late onset) diseases.

Acknowledgements

The authors want to thank the midwifes Patricia Bemelmans, Chantal Mulkens, Charlotte Peerboom and Ellen de Jong from the midwife practice Sittard, together with the midwifes from several practices surrounding Maastricht and the gynaecologists and residents gynaecology and obstetrics from the Maastricht University Medical Centre, Atrium hospital Heerlen and Orbis Medical Centre Sittard for their assistance in collecting placental tissues. We thank Marij Janssen and Marion Meijs for their administrative support, and Marijke Bras, Inge Schreurs, Sander Kleijkers, Roel Thijssen, Erika Timmer and Bieke Vanherle for their assistance in processing the samples.

References

Apostolidou S, Abu-Amero S, O'Donoghue K, Frost J, Olafsdottir O, Chavele KM, Whittaker JC, Loughna P, Stanier P,Moore GE. Elevated placental expression of the imprinted PHLDA2 gene is associated with low birth weight. *J Mol Med* 2007;**85**:(4): 379-387.

Barker DJ, Osmond C, Kajantie E,Eriksson JG. Growth and chronic disease: findings in the Helsinki Birth Cohort. *Ann Hum Biol* 2009;**36**:(5): 445-458.

Ceelen M, van Weissenbruch MM, Roos JC, Vermeiden JP, van Leeuwen FE,Delemarre-van de Waal HA. Body composition in children and adolescents born after in vitro fertilization or spontaneous conception. *J Clin Endocrinol Metab* 2007;**92**:(9): 3417-3423.

Ceelen M, van Weissenbruch MM, Vermeiden JP, van Leeuwen FE,Delemarre-van de Waal HA. Cardiometabolic differences in children born after in vitro fertilization: follow-up study. *J Clin Endocrinol Metab* 2008;**93**:(5): 1682-1688.

Diplas AI, Lambertini L, Lee MJ, Sperling R, Lee YL, Wetmur J,Chen J. Differential expression of imprinted genes in normal and IUGR human placentas. *Epigenetics* 2009;**4**:(4): 235-240.

Dixon WJ. Analysis of extreme values. *Annals of Mathematical Statistics* 1950;**21**: 488-506.

Dumoulin JC, Land JA, Van Montfoort AP, Nelissen EC, Coonen E, Derhaag JG, Schreurs IL, Dunselman GA, Kester AD, Geraedts JP, *et al.* Effect of in vitro culture of human embryos on birthweight of newborns. *Hum Reprod* 2010;**25**:(3): 605-612.

Eijssen LM, Jaillard M, Adriaens ME, Gaj S, de Groot PJ, Muller M,Evelo CT. User-friendly solutions for microarray quality control and pre-processing on ArrayAnalysis.org. *Nucleic Acids Res* 2013;**41**:(Web Server issue): W71-76.

Fauque P, Mondon F, Letourneur F, Ripoche MA, Journot L, Barbaux S, Dandolo L, Patrat C, Wolf JP, Jouannet P, *et al.* In vitro fertilization and embryo culture strongly impact the placental transcriptome in the mouse model. *PLoS One* 2010;**5**:(2): e9218.

Feng C, Tian S, Zhang Y, He J, Zhu XM, Zhang D, Sheng JZ,Huang HF. General imprinting status is stable in assisted reproduction-conceived offspring. *Fertil Steril* 2011;**96**:(6): 1417-1423 e1419.

Fortier AL, Lopes FL, Darricarrere N, Martel J,Trasler JM. Superovulation alters the expression of imprinted genes in the midgestation mouse placenta. *Hum Mol Genet* 2008;**17**:(11): 1653-1665.

Fraga MF, Ballestar E, Paz MF, Ropero S, Setien F, Ballestar ML, Heine-Suner D, Cigudosa JC, Urioste M, Benitez J, *et al.* Epigenetic differences arise during the lifetime of monozygotic twins. *Proc Natl Acad Sci U S A* 2005;**102**:(30): 10604-10609.

Geuns E, De Rycke M, Van Steirteghem A,Liebaers I. Methylation imprints of the imprint control region of the SNRPN-gene in human gametes and preimplantation embryos. *Hum Mol Genet* 2003;**12**:(22): 2873-2879.

Grubbs F. Procedures for Detecting Outlying Observations in Samples. *Technometrics* 1969;**11**:(1): 1-21.

Haavaldsen C, Tanbo T,Eskild A. Placental weight in singleton pregnancies with and without assisted reproductive technology: a population study of 536,567 pregnancies. *Hum Reprod* 2012;**27**:(2): 576-582.

Hiura H, Okae H, Miyauchi N, Sato F, Sato A, Van De Pette M, John RM, Kagami M, Nakai K, Soejima H, *et al.* Characterization of DNA methylation errors in patients with imprinting disorders conceived by assisted reproduction technologies. *Hum Reprod* 2012;**27**:(8): 2541-2548.

Huntriss JD, Hemmings KE, Hinkins M, Rutherford AJ, Sturmey RG, Elder K,Picton HM. Variable imprinting of the MEST gene in human preimplantation embryos. *Eur J Hum Genet* 2013;**21**:(1): 40-47.

Iglewicz B,Hoaglin D (1993). How to detect and handle outliers, ASQC Quality Press.

Jirtle RL,Skinner MK. Environmental epigenomics and disease susceptibility. *Nat Rev Genet* 2007;**8**:(4): 253-262.

Katari S, Turan N, Bibikova M, Erinle O, Chalian R, Foster M, Gaughan JP, Coutifaris C,Sapienza C. DNA methylation and gene expression differences in children conceived in vitro or in vivo. *Hum Mol Genet* 2009;**18**:(20): 3769-3778.

Kawahara M, Morita S, Takahashi N,Kono T. Defining contributions of paternally methylated imprinted genes at the Igf2-H19 and Dlk1-Gtl2 domains to mouse placentation by transcriptomic analysis. *J Biol Chem* 2009;**284**:(26): 17751-17765.

Kelder T, van Iersel MP, Hanspers K, Kutmon M, Conklin BR, Evelo CT,Pico AR. WikiPathways: building research communities on biological pathways. *Nucleic Acids Res* 2011;**40**:(Database issue): D1301-1307.

Kumar N, Leverence J, Bick D,Sampath V. Ontogeny of growth-regulating genes in the placenta. *Placenta* 2012;**33**:(2): 94-99.

Lambertini L, Diplas AI, Lee MJ, Sperling R, Chen J,Wetmur J. A sensitive functional assay reveals frequent loss of genomic imprinting in human placenta. *Epigenetics* 2008;**3**:(5): 261-269.

Lewis A, Mitsuya K, Umlauf D, Smith P, Dean W, Walter J, Higgins M, Feil R,Reik W. Imprinting on distal chromosome 7 in the placenta involves repressive histone methylation independent of DNA methylation. *Nat Genet* 2004;**36**:(12): 1291-1295.

Livak KJ,Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001;**25**:(4): 402-408.

Mann MR, Lee SS, Doherty AS, Verona RI, Nolen LD, Schultz RM,Bartolomei MS. Selective loss of imprinting in the placenta following preimplantation development in culture. *Development* 2004;**131**:(15): 3727-3735.

Market-Velker BA, Fernandes AD,Mann MR. Side-by-side comparison of five commercial media systems in a mouse model: suboptimal in vitro culture interferes with imprint maintenance. *Biol Reprod* 2010;**83**:(6): 938-950.

McMinn J, Wei M, Schupf N, Cusmai J, Johnson EB, Smith AC, Weksberg R, Thaker HM,Tycko B. Unbalanced placental expression of imprinted genes in human intrauterine growth restriction. *Placenta* 2006;**27**:(6-7): 540-549.

Nelissen EC, Dumoulin JC, Daunay A, Evers JL, Tost J,van Montfoort AP. Placentas from pregnancies conceived by IVF/ICSI have a reduced DNA methylation level at the H19 and MEST differentially methylated regions. *Hum Reprod* 2013;**28**:(4): 1117-1126.

Nelissen EC, van Montfoort AP, Dumoulin JC,Evers JL. Epigenetics and the placenta. *Hum Reprod Update* 2011;**17**:(3): 397-417.

Ogino S, Kawasaki T, Brahmandam M, Yan L, Cantor M, Namgyal C, Mino-Kenudson M, Lauwers GY, Loda M,Fuchs CS. Sensitive sequencing method for KRAS mutation detection by Pyrosequencing. *J Mol Diagn* 2005;**7**:(3): 413-421.

Oliver VF, Miles HL, Cutfield WS, Hofman PL, Ludgate JL,Morison IM. Defects in imprinting and genome-wide DNA methylation are not common in the in vitro fertilization population. *Fertil Steril* 2012;**97**:(1): 147-153 e147.

Palinski W. Maternal-fetal cholesterol transport in the placenta: good, bad, and target for modulation. *Circ Res* 2009;**104**:(5): 569-571.

Pandey RR, Mondal T, Mohammad F, Enroth S, Redrup L, Komorowski J, Nagano T, Mancini-Dinardo D,Kanduri C. Kcnq1ot1 antisense noncoding RNA mediates lineage-specific transcriptional silencing through chromatin-level regulation. *Mol Cell* 2008;**32**:(2): 232-246.

Pandey S, Shetty A, Hamilton M, Bhattacharya S,Maheshwari A. Obstetric and perinatal outcomes in singleton pregnancies resulting from IVF/ICSI: a systematic review and meta-analysis. *Hum Reprod Update* 2012;**18**:(5): 485-503.

Prickett AR,Oakey RJ. A survey of tissue-specific genomic imprinting in mammals. *Mol Genet Genomics* 2012;**287**:(8): 621-630.

Puumala SE, Nelson HH, Ross JA, Nguyen RH, Damario MA,Spector LG. Similar DNA methylation levels in specific imprinting control regions in children conceived with and without assisted reproductive technology: a cross-sectional study. *BMC Pediatr* 2012;**12**: 33.

Rancourt RC, Harris HR, Barault L,Michels KB. The prevalence of loss of imprinting of H19 and IGF2 at birth. *Faseb J* 2013;**27**:(8): 3335-3343.

Rancourt RC, Harris HR,Michels KB. Methylation levels at imprinting control regions are not altered with ovulation induction or in vitro fertilization in a birth cohort. *Hum Reprod* 2012;**27**:(7): 2208-2216.

Redrup L, Branco MR, Perdeaux ER, Krueger C, Lewis A, Santos F, Nagano T, Cobb BS, Fraser P,Reik W. The long noncoding RNA Kcnq1ot1 organises a lineage-specific nuclear domain for epigenetic gene silencing. *Development* 2009;**136**:(4): 525-530.

Reik W,Walter J. Genomic imprinting: parental influence on the genome. *Nat Rev Genet* 2001;**2**:(1): 21-32.

Rivera RM, Stein P, Weaver JR, Mager J, Schultz RM,Bartolomei MS. Manipulations of mouse embryos prior to implantation result in aberrant expression of imprinted genes on day 9.5 of development. *Hum Mol Genet* 2008;**17**:(1): 1-14.

Rosner B. Percentage Points for a Generalized ESD Many-Outlier Procedure. *Technometrics* 1983;**25**:(2): 165-172.

Ryter SW,Tyrrell RM. The heme synthesis and degradation pathways: role in oxidant sensitivity. Heme oxygenase has both pro- and antioxidant properties. *Free Radic Biol Med* 2000;**28**:(2): 289-309.

Sakka SD, Loutradis D, Kanaka-Gantenbein C, Margeli A, Papastamataki M, Papassotiriou I,Chrousos GP. Absence of insulin resistance and low-grade inflammation despite early metabolic syndrome manifestations in children born after in vitro fertilization. *Fertil Steril* 2010;**94**:(5): 1693-1699.

Sandovici I, Hoelle K, Angiolini E,Constancia M. Placental adaptations to the maternal-fetal environment: implications for fetal growth and developmental programming. *Reprod Biomed Online* 2012;**25**:(1): 68-89.

Sato A, Otsu E, Negishi H, Utsunomiya T,Arima T. Aberrant DNA methylation of imprinted loci in superovulated oocytes. *Hum Reprod* 2007;**22**:(1): 26-35.

Scherrer U, Rimoldi SF, Rexhaj E, Stuber T, Duplain H, Garcin S, de Marchi SF, Nicod P, Germond M, Allemann Y, *et al.* Systemic and pulmonary vascular dysfunction in children conceived by assisted reproductive technologies. *Circulation* 2012;**125**:(15): 1890-1896.

Tabano S, Colapietro P, Cetin I, Grati FR, Zanutto S, Mando C, Antonazzo P, Pileri P, Rossella F, Larizza L, *et al.* Epigenetic modulation of the IGF2/H19 imprinted domain in human embryonic and extra-embryonic compartments and its possible role in fetal growth restriction. *Epigenetics* 2010;**5**:(4): 313-324.

Tierling S, Souren NY, Gries J, Loporto C, Groth M, Lutsik P, Neitzel H, Utz-Billing I, Gillessen-Kaesbach G, Kentenich H, *et al.* Assisted reproductive technologies do not enhance the variability of DNA methylation imprints in human. *J Med Genet* 2010;**47**:(6): 371-376.

Tost J,Gut IG. DNA methylation analysis by pyrosequencing. *Nat Protoc* 2007;**2**:(9): 2265-2275.

Turan N, Katari S, Gerson LF, Chalian R, Foster MW, Gaughan JP, Coutifaris C,Sapienza C. Inter- and intra-individual variation in allele-specific DNA methylation and gene expression in children conceived using assisted reproductive technology. *PLoS Genet* 2010;**6**:(7): e1001033.

Tycko B. Imprinted genes in placental growth and obstetric disorders. *Cytogenet Genome Res* 2006;**113**:(1-4): 271-278.

Tycko B,Morison IM. Physiological functions of imprinted genes. *J Cell Physiol* 2002;**192**:(3): 245-258.

van der Loo MPJ (2010). Distribution based outlier detection for univariate data. The Hague, Statistics Netherlands

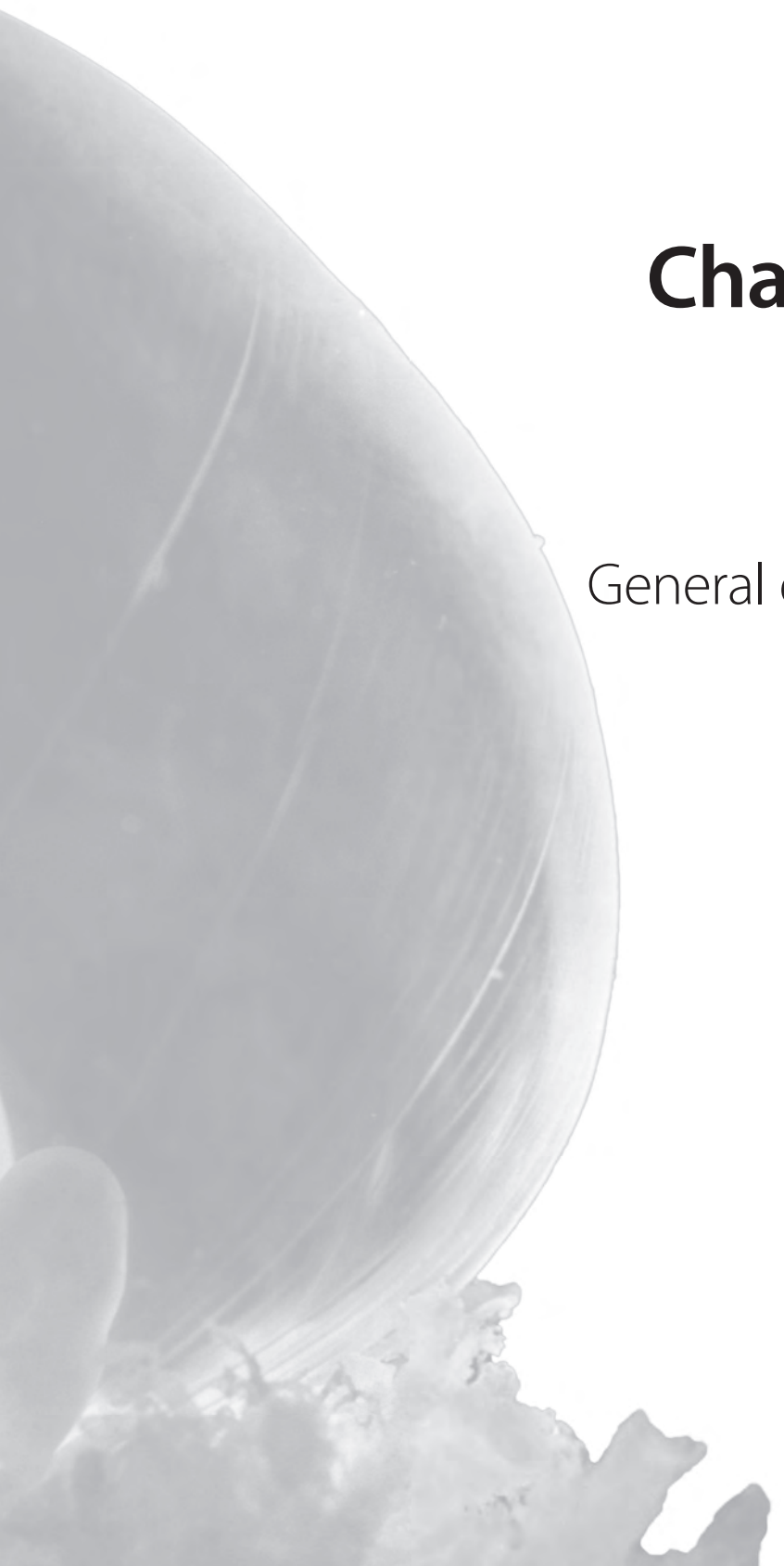
van Iersel MP, Kelder T, Pico AR, Hanspers K, Coort S, Conklin BR,Evelo C. Presenting and exploring biological pathways with PathVisio. *BMC Bioinformatics* 2008;**9**: 399.

van Montfoort AP, Hanssen LL, de Sutter P, Viville S, Geraedts JP,de Boer P. Assisted reproduction treatment and epigenetic inheritance. *Hum Reprod Update* 2012;**18**:(2): 171-197.

Wagschal A,Feil R. Genomic imprinting in the placenta. *Cytogenet Genome Res* 2006;**113**:(1-4): 90-98.

Wagschal A, Sutherland HG, Woodfine K, Henckel A, Chebli K, Schulz R, Oakey RJ, Bickmore WA,Feil R. G9a histone methyltransferase contributes to imprinting in the mouse placenta. *Mol Cell Biol* 2008;**28**:(3): 1104-1113.

Zhang Y, Cui Y, Zhou Z, Sha J, Li Y,Liu J. Altered global gene expressions of human placentae subjected to assisted reproductive technology treatments. *Placenta* 2010;**31**:(4): 251-258.



Chapter 7

General discussion

General Discussion

Although multiple studies have associated singletons conceived after ART with an adverse perinatal outcome (as described in the introduction), the underlying etiology of this association is as yet unknown. We hypothesized that culturing human embryos during the first few days of preimplantation development influences the perinatal outcome. Hereafter, we investigated whether ART has an effect on the epigenetic regulation of the human placenta.

The effect of culture medium on perinatal outcome

From animal studies it is clear that culture medium constituents are responsible for birthweight differences in offspring (as described in the introduction). However, in human ART there is still little knowledge of the effect of culture media on birthweight.

In the first part of this thesis, we investigated whether *in vitro* culture medium for human embryos during the first few days of preimplantation development affects perinatal outcome. We confirmed that the type of culture medium used had a significant effect on fetal development and mean birthweight (adjusted mean difference between two commercially available media, 112g). Our studies imply that ART-procedure related factors may be, at least in part, the cause of the adverse perinatal outcome. Following our studies, eight other groups compared different commercially available culture media of which three also found a difference in birthweight (Eaton *et al.*, 2012; Vergouw *et al.*, 2012; Carrasco *et al.*, 2013; Eskild *et al.*, 2013; Hassani *et al.*, 2013; Lin *et al.*, 2013; Lemmen *et al.*, 2014; Zhu *et al.*, 2014a). Carrasco *et al.* (2013) is the only study that compared the same media as we did. The difference was not significant, but points in the same direction as in our study, namely that *in vitro* culture in medium from Cook resulted in singletons with a lower mean birthweight adjusted for gestational age and gender. Three studies reported a difference in birthweight (Eskild *et al.*, 2013; Hassani *et al.*, 2013; Zhu *et al.*, 2014a). A large Norwegian retrospective population based cohort study compared three different culture media (Medicult Universal, Medicult ISM1 and Vitrolife G1™ PLUS) and investigated birthweight of singletons born after IVF using the different culture media types consecutively during the study period, whilst birthweight changes in the general population over time were taken into account (Eskild *et al.*, 2013). A small prospective randomized study from an

Iranian group showed significant higher birthweights in singletons conceived after culture in Medicult ISM1 compared with Vitrolife G1™ v5 (Hassani *et al.*, 2013). Recently, a Chinese group found that the protein source in culture medium influenced z-scores but not absolute birthweights in IVF singletons (Zhu *et al.*, 2014a). This retrospective study investigated two types of medium which were used alternately: Vitrolife G1™ v5 supplemented with 5mg/ml pharmaceutical human serum albumin solution versus the ready-to-use Vitrolife G1™ PLUS v5. Other studies comparing culture in, respectively, Vitrolife G1™ v3/Global/ G1™ v5 media, HTF/Sage Quinn's advantage protein plus media, Vitrolife G1™ v3/Cook K-SICM/Medicult ISM1, Vitrolife G5™/Global/Sage Quinn's advantage media and Cook K-SICM/Medicult Embryoassist media did not show any significant differences in mean birthweight between singletons cultured in the different media (Eaton *et al.*, 2012; Vergouw *et al.*, 2012; Carrasco *et al.*, 2013; Lin *et al.*, 2013; Lemmen *et al.*, 2014).

The results of all these studies should be taken with care since all, including ours, have limitations. First of all, so far no adequate RCT has been performed. Our study was an RCT with quasi-random sequence generation, as alternate allocation to one of two media was performed with two-sided allocation concealment and blinding, which closely approaches optimal randomization. All other studies had a retrospective, non-randomized design, except for the small prospective studies of Hassani *et al.* (2013) and Carrasco *et al.* (2013). Only the latter study randomized between two culture media identical to our study, but their sample size was established to investigate clinical pregnancy rates (Carrasco *et al.*, 2013). Secondly, different culture medium types have been compared and they were often used separately in time, which does not allow correction for changes in population characteristics or birthweight trends over time unless there is a control population. Thirdly, to detect small differences in birthweight, studies with large sample sizes are needed. A sample size calculation indicates that to be able to demonstrate a birthweight difference of only 100g with a standard deviation of 565 g, a power of 80% and a significance level of 0.05, an RCT including at least 4000 couples resulting in 1000 children considering an average IVF pregnancy rate of 25% per cycle, is necessary. Such an RCT is time consuming and costly. Last but not least, the focus of attention, also in our study, is often directed towards birthweight and preterm birth. These are feasible endpoints to assess the effect of ART on short and long term morbidity. However, birthweight is a surrogate marker that can be influenced by

numerous factors, thus requiring multiple regression analysis to control for all known possible confounders, like parity, BMI, maternal weight gain, smoking, alcohol consumption, pre-existing maternal disease, and duration and etiology of subfertility. The disadvantage of the retrospective study designs is that often not all of these parameters are recorded and thus cannot be corrected for. Unfortunately, in the Netherlands data about maternal weight gain during pregnancy, which is associated with fetal growth, are rarely recorded. On top of this, humans are genetically very diverse which makes comparisons even more difficult than for instance in genetically identical mouse models. Theoretically, preterm birth can also have an iatrogenic cause via more rigorous obstetric interventions in "precious" ART pregnancies. This however does not seem to be the case (Romundstad *et al.*, 2009). Low birthweight is related to gestational age and as such also associated with prematurity. The fetal growth trajectory may be a more informative endpoint. We have shown an effect of culture medium on fetal growth rate (Chapter 3). However, this endpoint includes sonographic parameters measured during pregnancy with possible intra- and interobserver variability and sonographic weight formulas are known to have a limited accuracy (Dudley, 2005). A more direct marker which measures the adverse effect of culture medium and ART on perinatal outcome in humans so far is lacking.

However, despite these limitations, there are more studies indicating that ART-procedure related factors are at least partially responsible for the adverse outcome in ART singletons. As described in the introduction, subfertility plays a significant role in this adverse perinatal outcome. Nevertheless, even in the same mother an ART singleton has a poorer outcome than its non-ART sibling, indicating a risk attributable to ART *per* se (Pinborg *et al.*, 2013). Interestingly, singletons born after FET have a better perinatal outcome compared with singletons born after fresh embryo transfer regarding low birthweight and preterm birth, but a worse perinatal outcome compared with singletons born after spontaneous conception. Singletons born after FET have an increased risk of being large for gestational age (LGA), exceeding that of singletons born after spontaneous conception (Sazonova *et al.*, 2012; Nakashima *et al.*, 2013; Wennerholm *et al.*, 2013). Even when one singleton sibling is born after fresh transfer and the other after FET, the higher mean birthweight in singletons born after FET remained even after adjustment for maternal age and birth order (Henningsen *et al.*, 2011). This implies that the cryopreservation techniques

in combination with other patient-unrelated factors like minimal ovarian stimulation or favourable endometrial effects, play a role in the developmental potential of the embryo and the further intra-uterine growth. Concerning the IVF/ICSI technique, a lower risk of preterm birth or low birthweight is seen in ICSI versus IVF singletons (Pinborg *et al.*, 2013), which could however be related to more fertile mothers in ICSI cases when compared with IVF cases. Another potential factor is the influence of the number of culture days. While there is currently no culture medium available which is truly optimized for human embryo development, it is fair to reason that culturing embryos for 5-6 days until the blastocyst stage should incur a greater risk than culturing them for 2-3 days. In a recent large Canadian ART register analysis, the risk of preterm birth (<37 weeks) was significantly increased in singletons after extended embryo culture (blastocyst stage transfer on Day 5/6) compared with cleavage stage (Day 3) transfer (Dar *et al.*, 2013). These findings are in agreement with two previous Swedish and American large cohort studies (Kallen *et al.*, 2010; Kalra *et al.*, 2012). Other recent studies found a significant effect of prolonged embryo culture (5-6 days) on birthweight and Z-scores of singletons (Makinen *et al.*, 2013; Zhu *et al.*, 2014b). These large studies support our findings although the effects of prolonged culture time might also be induced by other culture conditions like oxygen level, mineral oil, culture dish etcetera.

In humans experimental designs are limited compared with animal models for ethical reasons. For example, it is difficult to isolate the contribution of specific ART-procedure related factors, for instance the effects of hormonal stimulation from the effects of the IVF laboratory procedures, to the overall risk of an adverse perinatal outcome after ART. Therefore, some factors related to the ART itself remain inconclusive. For instance, hormonal stimulation leads to multiple follicular maturation and corpus lutea which modifies the endocrine profile, and may have a possible negative downstream effect on the endometrium (Pelinck *et al.*, 2010). Singletons after ovulation induction with IUI appear to have a better outcome than IVF singletons, which can be attributed to more aggressive ovarian stimulation regimens or again to a negative impact of the IVF laboratory procedures (Wang *et al.*, 2002; De Sutter *et al.*, 2005). Moreover, fresh embryo transfer after IVF with ovarian stimulation resulted in a significantly lower birthweight compared with IVF in a modified natural cycle or FET, both with less ovarian stimulation (Pelinck *et al.*, 2010; Nakashima *et al.*, 2013; Pinborg *et al.*, 2013). It appears that hormonal stimulation has a role in the

adverse perinatal outcome after ART. In contrast to this, several studies found no association between the total dose of gonadotrophins or the number of oocytes retrieved and obstetric outcome (Chung *et al.*, 2006; Griesinger *et al.*, 2008; Sazonova *et al.*, 2011). Possibly, hormone levels during ART, for instance estradiol, are more relevant than total dose of gonadotrophins or number of oocytes. Imudia *et al.* (2012) found that elevated peak serum E2 levels on the day of hCG administration during COH was associated with greater odds of developing pre-eclampsia and a SGA infant in singleton pregnancies, while there was no difference in the total dose of gonadotrophins (Imudia *et al.*, 2012).

In conclusion, our studies along with other studies have shown that ART-procedure related factors, beside subfertility, play a role in the adverse perinatal outcome in ART singletons. The causes of the poorer outcome in ART singletons are probably multifactorial with culture medium being at least one of the factors responsible.

The role of epigenetic deregulation of the placenta in the adverse perinatal outcome after ART

In the second part of the thesis, we investigated in humans whether ART has an effect on the epigenetic regulation of the placenta, which might be an explanation for the reported adverse perinatal outcomes. Chapter 4 highlights the importance of the placenta for intrauterine growth and development and the requirement of a proper epigenetic regulation of both imprinted and non-imprinted genes. We found a reduced DNA methylation level at the *H19* and *MEST* differentially methylated regions (DMRs), and an increased RNA expression of *H19* and *PHLDA2* in placentas from pregnancies conceived by IVF/ICSI when compared with placentas from spontaneous conception. These are all placental growth-related genes. However, there was no indication for more LOI in IVF/ICSI placental samples according to our quantitative allele-specific expression analysis. Furthermore, genome-wide mRNA expression analysis revealed 13 significantly overrepresented biological pathways, which are involved in metabolism, immune response, transmembrane signalling and cell cycle control. Most were upregulated in the IVF/ICSI placental samples.

Our studies have shown that human placentas display epigenetic disturbances after ART when compared with placentas from spontaneous pregnancies. It is unknown whether our found epigenetic effects are caused by culture medium as we investigated placentas from ART and spontaneous

pregnancies. However, in our culture medium study (Chapter 3) fβ-hCG measured at around 12 weeks' gestation was found to be significantly higher after culture in Vitrolife medium than in Cook medium. As hCG is produced by the syncytiotrophoblast cells, the reported birthweight difference could be caused by a placental effect.

Besides our studies, only a few others have focused on epigenetic deregulation after ART in the human placenta. In placentas after ART treatments, DNA methylation differences have been reported of imprinted and non-imprinted genes (Katari *et al.*, 2009; Rancourt *et al.*, 2012) and at the *H19/IGF2* imprinting control region (*H19 ICR1*) (Turan *et al.*, 2010). A correlation between *H19* hypomethylation and increased expression was, as in our study, reported by Rancourt *et al.* (2012), but not by Turan *et al.* (2010). Katari et al. (2009) showed that several of the genes whose expression differed between the *in vitro* and *in vivo* groups are known to affect adipocyte development and differentiation, insulin signalling, and obesity. A qualitatively poor study found no differences in gene expression of several imprinted genes (Katagiri *et al.*, 2010). Furthermore, another small study found an altered global gene expression in three term placentas after ART compared with three placentas of spontaneous pregnancies (Zhang *et al.*, 2010). They found 18 differentially expressed genes and classified them according to their role in biological processes in immune response, transmembrane transport, metabolism, oxidative stress, cell differentiation and other processes. These pathways are strikingly comparable to those found in our study. Although our findings are in agreement with the findings of these studies, it is difficult to translate these epigenetic alterations to their clinical significance, as often no reduced birthweight was found. Other placental epigenetic studies, not involving ART, have shown that an increased *PHLDA2* expression was found in the placenta of intrauterine growth restricted (IUGR) babies (McMinn *et al.*, 2006; Diplas *et al.*, 2009; Kumar *et al.*, 2012), and an association between elevated placental *PHLDA2* expression levels and low birthweight was described (Apostolidou *et al.*, 2007).

The epigenetic alterations in the placenta can be seen as placental adaptations to withstand environmental cues without compromising fetal supply or as a deregulation leading to adverse developmental programming of the fetus. Placental deregulation may either directly affect fetal development or indirectly by changing the maternal gestational physiology, for instance through an effect on maternal metabolism or gestational disease like gestational diabetes

and pre-eclampsia (Sandovici *et al.*, 2012). More and more, it has become clear that the placenta is not just a passive organ mediating maternal-fetal exchange, it is able to adapt to intrinsic and extrinsic factors.

In humans, an increased frequency of placenta-related problems is observed in IVF/ICSI derived pregnancies, such as an increased risk of hypertensive disorders of pregnancy, placenta praevia, abruption and third trimester vaginal bleeding (Pandey *et al.*, 2012). Also, enlarged placentas and high placental weight in relation to birthweight, which persisted after adjustment for length of gestation, offspring birthweight, parity, fetal sex, maternal age, pre-eclampsia and diabetes (Haavaldsen *et al.*, 2012), and a significantly higher mean placental thickness was found among pregnancies conceived by ART compared with spontaneous pregnancies (Joy *et al.*, 2012). By using transmission-electron microscopy, a small study revealed a mild alteration of the placental barrier in ART-derived placentas (Zhang *et al.*, 2011). They found degenerative alterations of the terminal villi, mainly in syncytiotrophoblasts, including a thicker placental barrier, decreased apical microvilli and increased multiple vacuoles, which may suggest an effect on transplacental transports and exchanges.

Other human studies performed in a variety of tissues, i.e. preimplantation embryo, umbilical cord blood, amnion membrane, cord, buccal smear and peripheral blood from ART infants, have shown conflicting data with respect to ART induced alterations in methylation and expression of genes (Geuns *et al.*, 2003; Tierling *et al.*, 2010; Feng *et al.*, 2011; Hiura *et al.*, 2012; Oliver *et al.*, 2012; Puumala *et al.*, 2012; Huntriss *et al.*, 2013). It is possible that the ART offspring is developmentally programmed by subtle epigenetic aberrations, which may be hard to detect due to normal variation unless very large groups are investigated. In none of these studies, an association between epigenetic disturbances and birthweight was found. This is not strange as birthweight is a surrogate marker for outcome, as explained earlier, and it does not exclude the possibility of developmental programming in ART offspring. Also, a recent study showed that mice fetuses displayed a rapid catch-up growth during the second half of gestation and no birthweight difference were seen between IVF and control animals, while in the IVF group a modified placental transport was found (Bloise *et al.*, 2012). This again indicates that birthweight is a poor marker for the adverse effect of ART. A more direct marker of epigenetic disturbance which truly denotes an adverse effect of ART in humans would be crucial. However, such a marker needs yet to be discovered. Moreover, there

are some methodological and biological limitations. These include varied methylation assays (sometimes limited to 1–2 CpGs), non-allelic analyses, missing controls, small sample sizes, oocyte, embryo, and tissue pooling, the use of discarded/failed human reproductive samples (non-fertilized oocytes, fragmented embryos or chorionic villus samples of human abortions and stillbirths), age (preimplantation, midgestation, term and childhood), and type of tissue (i.e. embryo, placenta and peripheral blood cells), type of infertility, as well as patient characteristics (parental age, smoking, and obesity). Lastly, accumulating evidence has shown the importance of histone methylation and non-coding RNAs rather than DNA methylation in imprinting maintenance in the mouse placenta (Lewis *et al.*, 2004; Wagschal *et al.*, 2006; Pandey *et al.*, 2008; Wagschal *et al.*, 2008; Redrup *et al.*, 2009). Also in human placental development, emerging evidence suggests that non-coding RNAs act as important gene regulators (Fu *et al.*, 2013; Buckberry *et al.*, 2014).

How can culture medium cause epigenetic alterations? A possible explanation could be through an *in vitro* culture-induced effect upon the cell metabolism of the embryo. For instance, Banrezes et al. found that the post-natal growth rates of mice could be increased or decreased by experimentally manipulating the redox potential and mitochondrial activity after fertilization in 1-cell embryos (Banrezes *et al.*, 2011). This suggests that a disturbed cell metabolism may lead to epigenetic reprogramming with postnatal phenotypic effects. Interestingly, a recent study showed that slower developing embryos were most similar to *in vivo* controls, while faster embryos displayed a greater embryo volume, cell number and a deregulation of imprinted en metabolic gene expression (Market Velker *et al.*, 2012).

Wider implications

There is a growing concern about the safety of ART. According to the developmental origins of health and disease (DOHaD) hypothesis, exposure to an abnormal environment in pregnancy (or even during the preimplantation period) can predispose the individual to chronic diseases later in life, such as hypertension, type 2 diabetes, coronary heart disease and stroke (Barker *et al.*, 2009). A plausible explanation for DOHaD is epigenetic deregulation, which can lead to adverse developmental programming of the fetus. Epigenetic changes can even be transmitted to future generations in animal models (Skinner *et al.*, 2010). This may imply a serious health risk for ART offspring worldwide.

Studies have shown that in utero exposure to religious fasting or a period of famine, such as during the Dutch Hunger Winter, can influence the size and efficacy of the placenta (Alwasel *et al.*, 2010; Roseboom *et al.*, 2011) and can cause persistent epigenetic differences in individuals conceived during the famine (Heijmans *et al.*, 2008). Indeed, evidence is accumulating in several species that both the fetal growth trajectory and its adaptive responses to the prenatal and postnatal environment may already be determined in the period around the time of conception (Kwong *et al.*, 2000; MacLaughlin *et al.*, 2005; Bloomfield *et al.*, 2006; Rumball *et al.*, 2009). Also, in the liver of both rats and sheep epigenetic alterations were seen after periconceptional maternal undernutrition (Kwong *et al.*, 2006; Sinclair *et al.*, 2007). Characteristics of the metabolic syndrome like vascular dysfunction, increased systolic blood pressure, increased fasting glucose level and skinfold thickness, have been reported in both human (Ceelen *et al.*, 2007; Ceelen *et al.*, 2008; Sakka *et al.*, 2010; Scherrer *et al.*, 2012) and mouse ART progeny (Fernandez-Gonzalez *et al.*, 2004; Watkins *et al.*, 2007; Scott *et al.*, 2010). Furthermore, ART induces vascular dysfunction in normal mice and also a shortened life span when challenged with a high-fat diet compared with control animals (Rexhaj *et al.*, 2013). They used culture media commonly used during ART in humans and culture time needed to obtain 2-cell embryos was sufficient to cause these changes. Progeny of male ART mice also exhibited vascular dysfunction and arterial hypertension suggesting underlying epigenetic modifications (Rexhaj *et al.*, 2013). Altogether, this suggests that the composition of IVF culture media is crucial during this sensitive period, as well as maternal nutrition during conception in spontaneous pregnancies.

In animals, phenotypic differences after ART are often more pronounced after *in vitro* culture, for instance the large offspring syndrome seen in sheep and cattle. In humans, these phenotypic differences, such as birthweight, appear less pronounced. However, the apparently normal newborn infants after ART might be carrying lifetime health risks. We found that the effect of culture medium during the first few days after fertilization on fetal growth and birthweight persisted during the first 2 years of life (Kleijkers *et al.*, 2014). We believe it is the responsibility of the IVF practitioners to inform patients of the potential risks.

Conclusion and future prospects

Worldwide, the number of IVF children increases every year, while the etiology of the adverse perinatal outcome of singletons conceived after ART is still incompletely understood. Subtle epigenetic alterations may account for the phenotypic differences, such as birthweight, or possibly long-term health risks. Safety of ART is of vital importance, however research in this field has been very limited so far. In order to promote embryo growth *in vitro*, commercial culture media are supplemented with growth factors, antioxidants, cytokines and vitamins, while full knowledge of human embryo requirements is lacking. Furthermore, the exact composition of commercial IVF culture media systems is usually proprietary. Culture media manufacturers need to disclose the exact composition of their media, including concentrations of components, to enable comparison of culture media. All IVF clinics need to consider the safety of future changes to culture media composition and laboratory protocols before introducing them into the clinical setting. Also, they should further investigate the effects of those already introduced. Most importantly, we believe that ART patients and their offspring should be fully informed of the potential risks.

Further research should focus on the comparison of culture media in terms of embryo viability, pregnancy rates and especially perinatal and long-term outcome. Currently, a multi-centre RCT is carried out in the Netherlands to further investigate the role of culture medium in human ART with respect to the above outcome parameters. However, the primary outcome of this RCT is live birth rate which means that this RCT is not powered to detect differences in birthweight (or even long-term side effects). Large RCTs are therefore needed to investigate commercially available culture media and possible additives (like growth factors) in order to prove their safety with regard to perinatal outcome and epigenetic modifications. To investigate epigenetic modifications, placental tissues from both humans and animals can be obtained from pregnancies after fresh and frozen embryo transfer, and compared with placentas from spontaneous pregnancies. In humans, these placentas can even be compared with placentas from spontaneous pregnancies in subfertile couples. An interesting alternative comparison group includes siblings conceived with and without ART. As fetal (trophoblast) DNA and mRNA is present in maternal blood, this could provide another research tool to investigate epigenetic aberrations in ART pregnancies. Herewith, high risk pregnancies could perhaps be detected

and monitored more closely in view of the adverse perinatal outcome. More fundamental research, which requires trophoblast cell lines and animal models, is needed to elucidate the involvement of histone modifications and non-coding RNAs in placental gene regulation. The molecular mechanisms that underlie placental adaptations in vivo are mostly still unknown and are a challenge to study given the complexity of the maternal-placental-fetal interactions. Also, further research of epigenetic regulation in gametes and preimplantation embryos is necessary. Finally, long term follow up studies of all children born after ART are needed to investigate the true risks for chronic diseases in later life.

References

Alwasel SH, Abotalib Z, Aljarallah JS, Osmond C, Alkharaz SM, Alhazza IM, Badr G,Barker DJ. Changes in placental size during Ramadan. *Placenta* 2010;**31**:(7): 607-610.

Apostolidou S, Abu-Amero S, O'Donoghue K, Frost J, Olafsdottir O, Chavele KM, Whittaker JC, Loughna P, Stanier P,Moore GE. Elevated placental expression of the imprinted PHLDA2 gene is associated with low birth weight. *J Mol Med* 2007;**85**:(4): 379-387.

Banrezes B, Sainte-Beuve T, Canon E, Schultz RM, Cancela J,Ozil JP. Adult body weight is programmed by a redox-regulated and energy-dependent process during the pronuclear stage in mouse. *PLoS One* 2011;**6**:(12): e29388.

Barker DJ, Osmond C, Kajantie E,Eriksson JG. Growth and chronic disease: findings in the Helsinki Birth Cohort. *Ann Hum Biol* 2009;**36**:(5): 445-458.

Bloise E, Lin W, Liu X, Simbulan R, Kolahi KS, Petraglia F, Maltepe E, Donjacour A,Rinaudo P. Impaired placental nutrient transport in mice generated by in vitro fertilization. *Endocrinology* 2012;**153**:(7): 3457-3467.

Bloomfield FH, Oliver MH,Harding JE. The late effects of fetal growth patterns. *Arch Dis Child Fetal Neonatal Ed* 2006;**91**:(4): F299-304.

Buckberry S, Bianco-Miotto T,Roberts CT. Imprinted and X-linked non-coding RNAs as potential regulators of human placental function. *Epigenetics* 2014;**9**:(1): 81-89.

Carrasco B, Boada M, Rodriguez I, Coroleu B, Barri PN,Veiga A. Does culture medium influence offspring birth weight? *Fertil Steril* 2013;**100**:(5): 1283-1288.

Ceelen M, van Weissenbruch MM, Roos JC, Vermeiden JP, van Leeuwen FE,Delemarre-van de Waal HA. Body composition in children and adolescents born after in vitro fertilization or spontaneous conception. *J Clin Endocrinol Metab* 2007;**92**:(9): 3417-3423.

Ceelen M, van Weissenbruch MM, Vermeiden JP, van Leeuwen FE,Delemarre-van de Waal HA. Cardiometabolic differences in children born after in vitro fertilization: follow-up study. *J Clin Endocrinol Metab* 2008;**93**:(5): 1682-1688.

Chung K, Coutifaris C, Chalian R, Lin K, Ratcliffe SJ, Castelbaum AJ, Freedman MF,Barnhart KT. Factors influencing adverse perinatal outcomes in pregnancies achieved through use of in vitro fertilization. *Fertil Steril* 2006;**86**:(6): 1634-1641.

Dar S, Librach CL, Gunby J, Bissonnette F,Cowan L. Increased risk of preterm birth in singleton pregnancies after blastocyst versus Day 3 embryo transfer: Canadian ART Register (CARTR) analysis. *Hum Reprod* 2013;**28**:(4): 924-928.

De Sutter P, Veldeman L, Kok P, Szymczak N, Van der Elst J,Dhont M. Comparison of outcome of pregnancy after intra-uterine insemination (IUI) and IVF. *Hum Reprod* 2005;**20**:(6): 1642-1646.

Diplas AI, Lambertini L, Lee MJ, Sperling R, Lee YL, Wetmur J,Chen J. Differential expression of imprinted genes in normal and IUGR human placentas. *Epigenetics* 2009;**4**:(4): 235-240.

Dudley NJ. A systematic review of the ultrasound estimation of fetal weight. *Ultrasound Obstet Gynecol* 2005;**25**:(1): 80-89.

Eaton JL, Lieberman ES, Stearns C, Chinchilla M,Racowsky C. Embryo culture media and neonatal birthweight following IVF. *Hum Reprod* 2012;**27**:(2): 375-379.

Eskild A, Monkerud L,Tanbo T. Birthweight and placental weight; do changes in culture media used for IVF matter? Comparisons with spontaneous pregnancies in the corresponding time periods. *Hum Reprod* 2013;**28**:(12): 3207-3214.

Feng C, Tian S, Zhang Y, He J, Zhu XM, Zhang D, Sheng JZ,Huang HF. General imprinting status is stable in assisted reproduction-conceived offspring. *Fertil Steril* 2011;**96**:(6): 1417-1423 e1419.

Fernandez-Gonzalez R, Moreira P, Bilbao A, Jimenez A, Perez-Crespo M, Ramirez MA, Rodriguez De Fonseca F, Pintado B,Gutierrez-Adan A. Long-term effect of in vitro culture of mouse embryos with serum on mRNA expression of imprinting genes, development, and behavior. *Proc Natl Acad Sci U S A* 2004;**101**:(16): 5880-5885.

Fu G, Brkic J, Hayder H,Peng C. MicroRNAs in Human Placental Development and Pregnancy Complications. *Int J Mol Sci* 2013;**14**:(3): 5519-5544.

Geuns E, De Rycke M, Van Steirteghem A,Liebaers I. Methylation imprints of the imprint control region of the SNRPN-gene in human gametes and preimplantation embryos. *Hum Mol Genet* 2003;**12**:(22): 2873-2879.

Griesinger G, Kolibianakis EM, Diedrich K,Ludwig M. Ovarian stimulation for IVF has no quantitative association with birthweight: a registry study. *Hum Reprod* 2008;**23**:(11): 2549-2554.

Haavaldsen C, Tanbo T,Eskild A. Placental weight in singleton pregnancies with and without assisted reproductive technology: a population study of 536,567 pregnancies. *Hum Reprod* 2012;**27**:(2): 576-582.

Hassani F, Eftekhari-Yazdi P, Karimian L, Rezazadeh Valojerdi M, Movaghar B, Fazel M, Fouladi HR, Shabani F,Johansson L. The Effects of ISM1 Medium on Embryo Quality and Outcomes of IVF/ICSI Cycles. *Int J Fertil Steril* 2013;**7**:(2): 108-115.

Heijmans BT, Tobi EW, Stein AD, Putter H, Blauw GJ, Susser ES, Slagboom PE,Lumey LH. Persistent epigenetic differences associated with prenatal exposure to famine in humans. *Proc Natl Acad Sci U S A* 2008;**105**:(44): 17046-17049.

Henningsen AK, Pinborg A, Lidegaard O, Vestergaard C, Forman JL,Andersen AN. Perinatal outcome of singleton siblings born after assisted reproductive technology and spontaneous conception: Danish national sibling-cohort study. *Fertil Steril* 2011;**95**:(3): 959-963.

Hiura H, Okae H, Miyauchi N, Sato F, Sato A, Van De Pette M, John RM, Kagami M, Nakai K, Soejima H*, et al.* Characterization of DNA methylation errors in patients with imprinting disorders conceived by assisted reproduction technologies. *Hum Reprod* 2012;**27**:(8): 2541-2548.

Huntriss JD, Hemmings KE, Hinkins M, Rutherford AJ, Sturmey RG, Elder K,Picton HM. Variable imprinting of the MEST gene in human preimplantation embryos. *Eur J Hum Genet* 2013;**21**:(1): 40-47.

Imudia AN, Awonuga AO, Doyle JO, Kaimal AJ, Wright DL, Toth TL,Styer AK. Peak serum estradiol level during controlled ovarian hyperstimulation is associated with increased risk of small for gestational age and preeclampsia in singleton pregnancies after in vitro fertilization. *Fertil Steril* 2012;**97**:(6): 1374-1379.

Joy J, Gannon C, McClure N,Cooke I. Is assisted reproduction associated with abnormal placentation? *Pediatr Dev Pathol* 2012;**15**:(4): 306-314.

Kallen B, Finnstrom O, Lindam A, Nilsson E, Nygren KG,Olausson PO. Blastocyst versus cleavage stage transfer in in vitro fertilization: differences in neonatal outcome? *Fertil Steril* 2010;**94**:(5): 1680-1683.

Kalra SK, Ratcliffe SJ, Barnhart KT,Coutifaris C. Extended embryo culture and an increased risk of preterm delivery. *Obstet Gynecol* 2012;**120**:(1): 69-75.

Katagiri Y, Aoki C, Tamaki-Ishihara Y, Fukuda Y, Kitamura M, Matsue Y, So A,Morita M. Effects of assisted reproduction technology on placental imprinted gene expression. *Obstet Gynecol Int* 2010;**2010**.

Katari S, Turan N, Bibikova M, Erinle O, Chalian R, Foster M, Gaughan JP, Coutifaris C,Sapienza C. DNA methylation and gene expression differences in children conceived in vitro or in vivo. *Hum Mol Genet* 2009;**18**:(20): 3769-3778.

Kleijkers SH, van Montfoort AP, Smits LJ, Viechtbauer W, Roseboom TJ, Nelissen EC, Coonen E, Derhaag JG, Bastings L, Schreurs IE*, et al.* IVF culture medium affects post-natal weight in humans during the first 2 years of life. *Hum Reprod* 2014;**29**:(4): 661-669.

Kumar N, Leverence J, Bick D,Sampath V. Ontogeny of growth-regulating genes in the placenta. *Placenta* 2012;**33**:(2): 94-99.

Kwong WY, Miller DJ, Ursell E, Wild AE, Wilkins AP, Osmond C, Anthony FW,Fleming TP. Imprinted gene expression in the rat embryo-fetal axis is altered in response to periconceptional maternal low protein diet. *Reproduction* 2006;**132**:(2): 265-277.

Kwong WY, Wild AE, Roberts P, Willis AC,Fleming TP. Maternal undernutrition during the preimplantation period of rat development causes blastocyst abnormalities and programming of postnatal hypertension. *Development* 2000;**127**:(19): 4195-4202.

Lemmen JG, Pinborg A, Rasmussen S,Ziebe S. Birthweight distribution in ART singletons resulting from embryo culture in two different culture media compared with the national population. *Hum Reprod* 2014.

Lewis A, Mitsuya K, Umlauf D, Smith P, Dean W, Walter J, Higgins M, Feil R,Reik W. Imprinting on distal chromosome 7 in the placenta involves repressive histone methylation independent of DNA methylation. *Nat Genet* 2004;**36**:(12): 1291-1295.

Lin S, Li M, Lian Y, Chen L,Liu P. No effect of embryo culture media on birthweight and length of newborns. *Hum Reprod* 2013;**28**:(7): 1762-1767.

MacLaughlin SM, Walker SK, Roberts CT, Kleemann DO,McMillen IC. Periconceptional nutrition and the relationship between maternal body weight changes in the periconceptional period and fetoplacental growth in the sheep. *J Physiol* 2005;**565**:(Pt 1): 111-124.

Makinen S, Soderstrom-Anttila V, Vainio J, Suikkari AM,Tuuri T. Does long in vitro culture promote large for gestational age babies? *Hum Reprod* 2013;**28**:(3): 828-834.

Market Velker BA, Denomme MM,Mann MR. Loss of genomic imprinting in mouse embryos with fast rates of preimplantation development in culture. *Biol Reprod* 2012;**86**:(5): 143, 141-116.

McMinn J, Wei M, Schupf N, Cusmai J, Johnson EB, Smith AC, Weksberg R, Thaker HM,Tycko B. Unbalanced placental expression of imprinted genes in human intrauterine growth restriction. *Placenta* 2006;**27**:(6-7): 540-549.

Nakashima A, Araki R, Tani H, Ishihara O, Kuwahara A, Irahara M, Yoshimura Y, Kuramoto T, Saito H, Nakaza A, *et al*. Implications of assisted reproductive technologies on term singleton birth weight: an analysis of 25,777 children in the national assisted reproduction registry of Japan. *Fertil Steril* 2013;**99**:(2): 450-455.

Oliver VF, Miles HL, Cutfield WS, Hofman PL, Ludgate JL,Morison IM. Defects in imprinting and genome-wide DNA methylation are not common in the in vitro fertilization population. *Fertil Steril* 2012;**97**:(1): 147-153 e147.

Pandey RR, Mondal T, Mohammad F, Enroth S, Redrup L, Komorowski J, Nagano T, Mancini-Dinardo D,Kanduri C. Kcnq1ot1 antisense noncoding RNA mediates lineage-specific transcriptional silencing through chromatin-level regulation. *Mol Cell* 2008;**32**:(2): 232-246.

Pandey S, Shetty A, Hamilton M, Bhattacharya S,Maheshwari A. Obstetric and perinatal outcomes in singleton pregnancies resulting from IVF/ICSI: a systematic review and meta-analysis. *Hum Reprod Update* 2012;**18**:(5): 485-503.

Pelinck MJ, Hadders-Algra M, Haadsma ML, Nijhuis WL, Kiewiet SM, Hoek A, Heineman MJ,Middelburg KJ. Is the birthweight of singletons born after IVF reduced by ovarian stimulation or by IVF laboratory procedures? *Reprod Biomed Online* 2010;**21**:(2): 245-251.

Pinborg A, Wennerholm UB, Romundstad LB, Loft A, Aittomaki K, Soderstrom-Anttila V, Nygren KG, Hazekamp J,Bergh C. Why do singletons conceived after assisted reproduction technology have adverse perinatal outcome? Systematic review and meta-analysis. *Hum Reprod Update* 2013;**19**:(2): 87-104.

Puumala SE, Nelson HH, Ross JA, Nguyen RH, Damario MA,Spector LG. Similar DNA methylation levels in specific imprinting control regions in children conceived with and without assisted reproductive technology: a cross-sectional study. *BMC Pediatr* 2012;**12**: 33.

Rancourt RC, Harris HR,Michels KB. Methylation levels at imprinting control regions are not altered with ovulation induction or in vitro fertilization in a birth cohort. *Hum Reprod* 2012;**27**:(7): 2208-2216.

Redrup L, Branco MR, Perdeaux ER, Krueger C, Lewis A, Santos F, Nagano T, Cobb BS, Fraser P,Reik W. The long noncoding RNA Kcnq1ot1 organises a lineage-specific nuclear domain for epigenetic gene silencing. *Development* 2009;**136**:(4): 525-530.

Rexhaj E, Paoloni-Giacobino A, Rimoldi SF, Fuster DG, Anderegg M, Somm E, Bouillet E, Allemann Y, Sartori C,Scherrer U. Mice generated by in vitro fertilization exhibit vascular dysfunction and shortened life span. *J Clin Invest* 2013;**123**:(12): 5052-5060.

Romundstad LB, Romundstad PR, Sunde A, von During V, Skjaerven R,Vatten LJ. Assisted fertilization and breech delivery: risks and obstetric management. *Hum Reprod* 2009;**24**:(12): 3205-3210.

Roseboom TJ, Painter RC, de Rooij SR, van Abeelen AF, Veenendaal MV, Osmond C,Barker DJ. Effects of famine on placental size and efficiency. *Placenta* 2011;**32**:(5): 395-399.

Rumball CW, Bloomfield FH, Oliver MH,Harding JE. Different periods of periconceptional undernutrition have different effects on growth, metabolic and endocrine status in fetal sheep. *Pediatr Res* 2009;**66**:(6): 605-613.

Sakka SD, Loutradis D, Kanaka-Gantenbein C, Margeli A, Papastamataki M, Papassotiriou I,Chrousos GP. Absence of insulin resistance and low-grade inflammation despite early metabolic syndrome manifestations in children born after in vitro fertilization. *Fertil Steril* 2010;**94**:(5): 1693-1699.

Sandovici I, Hoelle K, Angiolini E,Constancia M. Placental adaptations to the maternal-fetal environment: implications for fetal growth and developmental programming. *Reprod Biomed Online* 2012;**25**:(1): 68-89.

Sazonova A, Kallen K, Thurin-Kjellberg A, Wennerholm UB,Bergh C. Factors affecting obstetric outcome of singletons born after IVF. *Hum Reprod* 2011;**26**:(10): 2878-2886.

Sazonova A, Kallen K, Thurin-Kjellberg A, Wennerholm UB,Bergh C. Obstetric outcome in singletons after in vitro fertilization with cryopreserved/thawed embryos. *Hum Reprod* 2012;**27**:(5): 1343-1350.

Scherrer U, Rimoldi SF, Rexhaj E, Stuber T, Duplain H, Garcin S, de Marchi SF, Nicod P, Germond M, Allemann Y, *et al.* Systemic and pulmonary vascular dysfunction in children conceived by assisted reproductive technologies. *Circulation* 2012;**125**:(15): 1890-1896.

Scott KA, Yamazaki Y, Yamamoto M, Lin Y, Melhorn SJ, Krause EG, Woods SC, Yanagimachi R, Sakai RR,Tamashiro KL. Glucose parameters are altered in mouse offspring produced by assisted reproductive technologies and somatic cell nuclear transfer. *Biol Reprod* 2010;**83**:(2): 220-227.

Sinclair KD, Allegrucci C, Singh R, Gardner DS, Sebastian S, Bispham J, Thurston A, Huntley JF, Rees WD, Maloney CA, *et al.* DNA methylation, insulin resistance, and blood pressure in offspring determined by maternal periconceptional B vitamin and methionine status. *Proc Natl Acad Sci U S A* 2007;**104**:(49): 19351-19356.

Skinner MK, Manikkam M,Guerrero-Bosagna C. Epigenetic transgenerational actions of environmental factors in disease etiology. *Trends Endocrinol Metab* 2010;**21**:(4): 214-222.

Tierling S, Souren NY, Gries J, Loporto C, Groth M, Lutsik P, Neitzel H, Utz-Billing I, Gillessen-Kaesbach G, Kentenich H, *et al.* Assisted reproductive technologies do not enhance the variability of DNA methylation imprints in human. *J Med Genet* 2010;**47**:(6): 371-376.

Turan N, Katari S, Gerson LF, Chalian R, Foster MW, Gaughan JP, Coutifaris C,Sapienza C. Inter- and intra-individual variation in allele-specific DNA methylation and gene expression in children conceived using assisted reproductive technology. *PLoS Genet* 2010;**6**:(7): e1001033.

Vergouw CG, Kostelijk EH, Doejaaren E, Hompes PG, Lambalk CB,Schats R. The influence of the type of embryo culture medium on neonatal birthweight after single embryo transfer in IVF. *Hum Reprod* 2012;**27**:(9): 2619-2626.

Wagschal A,Feil R. Genomic imprinting in the placenta. *Cytogenet Genome Res* 2006;**113**:(1-4): 90-98.

Wagschal A, Sutherland HG, Woodfine K, Henckel A, Chebli K, Schulz R, Oakey RJ, Bickmore WA,Feil R. G9a histone methyltransferase contributes to imprinting in the mouse placenta. *Mol Cell Biol* 2008;**28**:(3): 1104-1113.

Wang JX, Norman RJ,Kristiansson P. The effect of various infertility treatments on the risk of preterm birth. *Hum Reprod* 2002;**17**:(4): 945-949.

Watkins AJ, Platt D, Papenbrock T, Wilkins A, Eckert JJ, Kwong WY, Osmond C, Hanson M,Fleming TP. Mouse embryo culture induces changes in postnatal phenotype including raised systolic blood pressure. *Proc Natl Acad Sci U S A* 2007;**104**:(13): 5449-5454.

Wennerholm UB, Henningsen AK, Romundstad LB, Bergh C, Pinborg A, Skjaerven R, Forman J, Gissler M, Nygren KG,Tiitinen A. Perinatal outcomes of children born after frozen-thawed embryo transfer: a Nordic cohort study from the CoNARTaS group. *Hum Reprod* 2013.

Zhang Y, Cui Y, Zhou Z, Sha J, Li Y,Liu J. Altered global gene expressions of human placentae subjected to assisted reproductive technology treatments. *Placenta* 2010;**31**:(4): 251-258.

Zhang Y, Zhao W, Jiang Y, Zhang R, Wang J, Li C, Zhao H, Gao L, Cui Y, Zhou Z, *et al.* Ultrastructural study on human placentae from women subjected to assisted reproductive technology treatments. *Biol Reprod* 2011;**85**:(3): 635-642.

Zhu J, Li M, Chen L, Liu P,Qiao J. The protein source in embryo culture media influences birthweight: a comparative study between G1 v5 and G1-PLUS v5. *Hum Reprod* 2014a.

Zhu J, Lin S, Li M, Chen L, Lian Y, Liu P,Qiao J. Effect of in vitro culture period on birthweight of singleton newborns. *Hum Reprod* 2014b;**29**:(3): 448-454.

Chapter 8

Valorisation

Why is this thesis relevant?

In this valorisation paragraph, I want to consider the potential impact of our research and to valorise what is of societal and economic value.

Relevance

The number of IVF children increases every year, while the etiology of the adverse perinatal outcome of singletons conceived after assisted reproduction technology (ART) is still incompletely understood. In humans, these phenotypic differences, such as birthweight, appear less pronounced than in animals. However, the apparently normal newborn infants after ART might be carrying lifetime health risks. Safety of ART is of vital importance, however research in this field has been very limited so far. We have shown that the type of culture medium used has a significant effect on fetal development and birthweight. Moreover, human placentas display epigenetic disturbances after ART when compared to placentas from spontaneous pregnancies.

Although ART has led to substantial knowledge of human embryology, it has also turned into a multi-million dollar industry. Worldwide, ART is mainly provided in the private health care sector. IVF clinics want to deliver as many healthy babies as possible. But as in some countries success rates of IVF clinics are published, ranking ART clinics according to their success rates in league tables could increase the pressure to use the latest technology in order to achieve the best results. IVF patients are very www-literate, so ART clinics may also be under strong pressure from patients to go ahead with innovations if expected benefits were already set out on the web or in the press. In ART there are many examples of new technologies and methods which have been introduced in clinical practice without appropriate evidence-base to show that the procedure is safe and beneficial to the patient, that it is cost-effective, and that its benefits outweigh its potential harms, for instance DHEA administration to 'rejuvenate' old eggs, time-lapse monitoring of embryo development, endometrial 'scratching', assisted hatching, *in vitro* maturation, blastocyst transfer, vitrification and preimplantation genetic screening (PGS) (Harper *et al.*, 2012). Furthermore, changes to culture media composition (supplementation with growth factors, antioxidants, cytokines and vitamins), stimulation regimens and laboratory protocols are often established internationally without adequate validation. "Assisted reproduction has been technologically driven rather than evidence

based. Treatment tends to be empirical, and existing evidence can be ignored in favour of novel interventions" (Bhattacharya *et al.*, 2001).

Target groups

The results of this thesis are interesting for IVF professionals, culture medium manufacturers, pharmaceutical and other industries, health economists and health care providers/insurers, politicians and the European Society of Human Reproduction and Embryology (ESHRE). Together we need to determine what the best course of action is. Above all, our research results are of interest to patients. What do patients want? Why do they accept or even insist on receiving treatments without any evidence? Are patients aware of the adverse outcome after ART or of the possible long-term health effects? Do they care? Infertility couples belong to a very vulnerable group. They will do everything to achieve their goal, a baby. They should not be exploited.

Activities/innovation

All our results have been published in high-ranking scientific research journals. We have had the opportunity to discuss our findings nationally and internationally to gain more attention for this important topic. This has led to more and more research groups worldwide investigating the effect of culture medium on human perinatal outcome. In the Netherlands, a multi-centre trial has been initiated comparing two commercially available culture media with respect to live birth rate and perinatal outcome. We played a role in the formation of the ESHRE working group on culture media with members from the special interest groups Embryology, Safety and Quality in ART, and Genetics. This working group held meetings with the largest ART culture media producers to encourage constructive co-operation over transparency, composition and quality control parameters. Regular expert meetings for instance organised by ESHRE, should raise global awareness and the development of recommendations and official guidelines.

The possibility that media and other culture conditions are partly responsible for an adverse perinatal outcome in IVF children should not be ignored. The extend of this adverse outcome however is still uncertain. Therefore, larger studies are required to investigate the etiology of this adverse perinatal outcome. Culture media manufacturers need to disclose the exact composition of their media, including concentrations of components, to enable a better comparison of culture media.

Schedule and implementation

IVF professionals need to consider the safety of future changes to culture media composition, stimulation regimens and laboratory protocols before introducing them into the clinical setting. New technologies should be evaluated for effectiveness, safety and cost-effectiveness. Also, they should further investigate the effects of those technologies that have already been introduced. We need more relevant preclinical testing, preferably using a more appropriate test than the mouse embryo assay. Subsequently, human embryos should be made available for research, and research should be performed on human eggs/sperm or embryos donated for research. This should be followed by large clinical trials with follow-up of IVF pregnancies and children. Innovation is important, however we need to be critical before introducing these innovations in human ART.

According to EU regulations, culture media intended for use in the IVF process to support the growth and/or storage of embryos are generally to be considered as Class III medical devices (EU Manual, 2014). These medical devices must meet certain essential requirements that are set out in the Medical Devices Directive 93/42/EEC and further explained in an EU Guideline (2014). In these guidelines it is stated that culture media must be CE (European Conformity) marked and that pre-clinical testing (including tests for genotoxicity, carcinogenicity and reproductive toxicity) should be performed in order to evaluate the risk due to the IVF product (e.g. culture media). Furthermore, manufacturers of such medical devices must perform pre-clinical and clinical evaluations and should plan a post-market clinical follow-up programme in order to provide the clinical safety and performance data on the use of the culture medium in clinical practice.

In view of the results described in this thesis, we hope to achieve more awareness for the existing legislation and most importantly its clinical implementation. Tighter regulation of the production of IVF culture media via national and European legislation is required to eventually disclose the exact composition of media.

Lastly, we believe that ART patients and their offspring should be fully informed of the adverse outcome after ART and the possible long-term health effects. A patient-preference investigation could enhance our knowledge on what patients want with regard to this very important topic.

References

Bhattacharya S, Hamilton MP, Shaaban M, Khalaf Y, Seddler M, Ghobara T, Braude P, Kennedy R, Rutherford A, Hartshorne G, *et al.* Conventional in-vitro fertilisation versus intracytoplasmic sperm injection for the treatment of non-male-factor infertility: a randomised controlled trial. *Lancet* 2001;**357**:(9274): 2075-2079.

Harper J, Magli MC, Lundin K, Barratt CL,Brison D. When and how should new technology be introduced into the IVF laboratory? *Hum Reprod* 2012;**27**:(2): 303-313.

Chapter 9

Summary

Summary

Chapter 1 provides a general introduction, as well as the outline of this thesis. The etiology of the adverse perinatal outcome of singletons conceived after assisted reproduction technology (ART) is still incompletely understood, even after all these years of ART use. In humans, these phenotypic differences, such as altered birthweight, appear less pronounced than in animals. However, the apparently normal newborn infants after ART might be carrying lifetime health risks. Although safety of ART is of vital importance, research in this field has been very limited so far. This thesis focuses on the effects of ART in humans on adverse perinatal outcome. We wished to gain more insight into its etiology and find ways to improve the safety of ART in the future. The first aim of the thesis was to investigate whether *in vitro* culture of human embryos during the first few days of preimplantation development affects perinatal outcome. The second aim of the thesis was to evaluate whether ART has an effect on the epigenetic regulation of the human placenta.

In **Chapter 2,** we evaluated the effect of *in vitro* culture medium on human perinatal outcome by comparing these outcomes in singleton pregnancies after alternative usage of two commercially available sequential media systems. Data on a cohort of 294 live born singletons conceived after transfer of fresh embryos during any of a patient's IVF treatment cycles, as well as data of 67 singletons conceived after frozen embryo transfer (FET) and of 88 children of 44 twin pregnancies after fresh transfer were analysed. *In vitro* culture in medium from Cook resulted in singletons after fresh transfer with a lower mean birthweight (adjusted mean difference, 112 grams, $P = 0.03$), and in more singletons with low birthweight (LBW) <2500g ($P = 0.006$) and LBW for GA ≥ 37 weeks ($P = 0.015$), when compared with singletons born after culture in medium from Vitrolife AB. Gestational age at birth was not related to the medium used (adjusted difference, 0.05 weeks, $P = 0.83$). Among twins in the Cook group, higher inter-twin mean birthweight disparity and birthweight discordance were found. Our findings supported our hypothesis that culture medium influences perinatal outcome of IVF singletons and twins. A similar trend was seen in case of singletons born after FET. These results indicate that *in vitro* culture might be an important factor explaining the poorer perinatal outcome after assisted reproduction technology (ART).

In **Chapter 3**, the impact of embryo culture media on fetal growth patterns, with particular focus on the onset of growth divergence, was investigated in a previously described cohort of singletons conceived after fresh transfer, as abnormal fetal growth patterns are a major risk factor for the development of chronic diseases in later life. We analysed ultrasound examinations at 8 (n = 290), 12 (n = 83) and 20 weeks' (n = 206) gestation and used first-trimester serum markers [pregnancy-associated plasma protein-A (PAPP-A) and free β-hCG]. At 8 weeks' gestation, there was no difference between crown-rump length-based and ovum retrieval-based gestational age (ΔGA) (n_{VL} = 163, n_C = 122, adjusted mean difference, -0.04 days, *P* = 0.84). At 12 weeks' gestation, ΔGA, nuchal translucency (multiples of median, MoM) and PAPP-A (MoM) did not differ between the study groups. Free β-hCG (MoM) ± SEM differed significantly (1.55 ± 0.19 in Vitrolife versus 1.06 ± 0.10 in Cook; *P* = 0.031). At 20 weeks' gestation, a more advanced GA, reflecting an increased fetal growth, was seen at ultrasound examination in the Vitrolife group (n = 115) when compared with the Cook group (n = 91). After adjustment for confounding factors, both the difference between GA based on three biparietal diameter dating formulas minus the actual (ovum retrieval based) GA (adjusted mean difference +1.14 days (*P* = 0.04), +1.14 days (*P* = 0.04) and +1.36 days (*P* = 0.048), as well as head circumference (HC) and trans-cerebellar diameter (TCD) were significantly higher in the Vitrolife group (HC_{vl} 177.3mm, HC_c 175.9mm, adjusted mean difference 1.8, *P* = 0.03; TCD_{vl} 20.5mm, TCD_c 20.2mm, adjusted mean difference 0.4, *P* = 0.008). In conclusion, differences in fetal development after culture of embryos in one of two IVF media were apparent as early as the second trimester of pregnancy. This study indicates that the embryonic environment is already important for fetal development.

Chapter 4 reviews the knowledge in the field of epigenetics in relation to placental development and function. Studies in both animals and humans have made it increasingly clear that proper epigenetic regulation of both imprinted and non-imprinted genes is important in placental development. Its disturbance, which can be caused by various environmental factors, can lead to abnormal placental development and function with possible consequences for maternal morbidity, fetal development and disease susceptibility in later life.

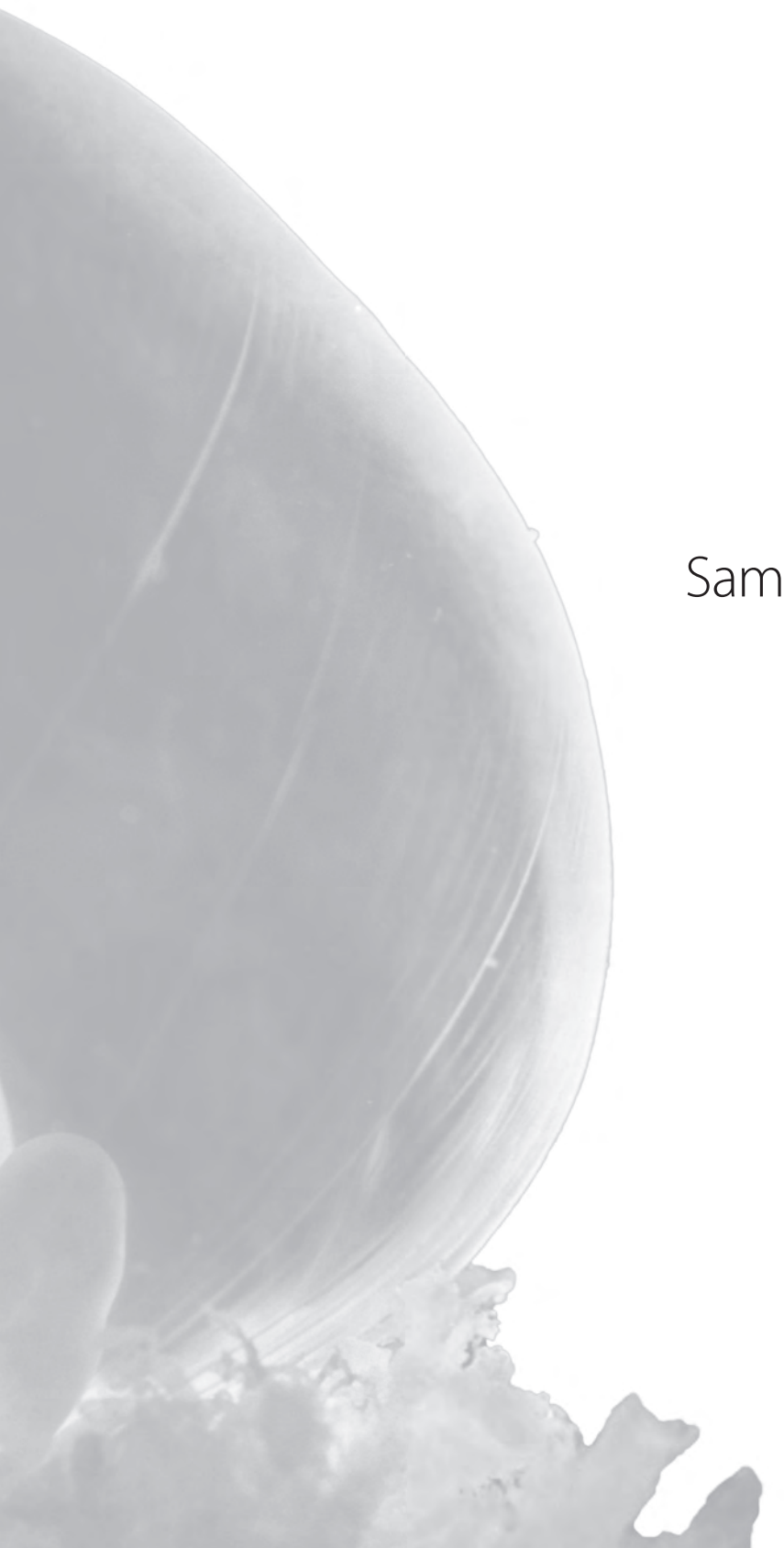
In **Chapter 5**, we investigated the possible effect of ART on the epigenetic regulation of human placentas. Placental tissue was collected from 35 spontaneously conceived pregnancies and 35 IVF/ICSI (5 IVF, 30 ICSI) derived pregnancies. We quantitatively analysed the DNA methylation patterns of a number of consecutive CpGs in the core regions of several differentially methylated regions (DMRs) involved in parent-of-origin specific expression of imprinted genes, since these are involved in placental and fetal growth and development. Both the promoter region of *MEST* isoform α and β and the 6th CTCF binding site within the *H19* DMR were significantly hypomethylated in the IVF/ICSI group. The phenomenon was consistently observed over all CpG sites analysed and not restricted to single CpG sites. The other primary and secondary DMRs were not affected. Expression of *H19* was increased in the IVF/ICSI group, while that of *IGF2* and *MEST* remained similar. The clinical effects of the observed placental hypomethylations on the developmental programming of the IVF/ICSI progeny, if any, are as yet unknown. Whether the hypomethylation is an adaptation of the placenta to maintain fetal supply and ameliorate the effects of environmental cues, or whether it is a deregulation leading to deranged developmental programming with or without increased vulnerability for disease, consistent with the developmental origins of health and disease (DOHaD) hypothesis, needs further investigation.

To further explore the effect of ART on the epigenetic regulation of the human placenta, in **Chapter 6** we carried out a microarray analysis (n=10 in each group) to investigate genome-wide which pathways are deregulated in IVF/ICSI placentas. The study revealed 13 significantly overrepresented biological pathways involved in metabolism, immune response, transmembrane signalling and cell cycle control, which were mostly up-regulated in the IVF/ICSI placental samples. Further, regarding the importance of imprinted genes in placental function, special attention was given to several growth-related imprinted genes (*H19, IGF2, PHLDA2, CDKN1C, MEST* isoform *α* and β) by analysing the expression in a larger number of samples using quantitative real-time PCR (qPCR) after standardisation against three housekeeping genes (*SDHA, YWHAZ* and *TBP*). For the genes that were differentially expressed, a quantitative allele-specific expression analysis was performed to investigate loss of imprinting (LOI), independent of the mechanism of imprinting. In this prospective cohort study, respectively, 81 and 105 placentas from IVF/ICSI and control

pregnancies remained for analysis after applying several exclusion criteria (i.e. preterm birth or stillbirth, no placental samples, pregnancy complications or birth defects). Saliva samples from both parents were used to distinguish parental alleles. Both *H19* and *PHLDA2* showed a significant change in gene expression, respectively, a 1.3-fold ($P = 0.033$) and 1.5-fold ($P = 0.002$) increase in mRNA expression in the IVF/ICSI versus control group. However, we found no indication that this increased expression is due to an increased frequency of LOI in IVF/ICSI placental samples. Whether these epigenetic alterations in placentas from pregnancies conceived by IVF/ICSI might be connected to an adverse perinatal outcome after IVF remains unknown. However, it is possible that these differences affect fetal development and long-term patterns of gene expression, as well as maternal gestational physiology.

Chapter 7 presents a discussion of the findings of this thesis within the context of recent literature. Conclusions of this thesis, wider implications and future prospects are given.

Chapter 8 provides a valorisation paragraph to consider the potential impact of our research and to valorise what is of societal and economic value.



Samenvatting

Samenvatting

Hoofdstuk 1 geeft een algemene inleiding alsook een overzicht van het proefschrift. De etiologie van de slechtere perinatale uitkomst van eenlingen verwekt na geassisteerde voortplanting (ART) is nog onvolledig bekend, zelfs na al die jaren van toepassing. Bij de mens lijken de fenotypische verschillen, zoals een veranderd geboortegewicht, minder uitgesproken dan bij dieren. Echter de ogenschijnlijk normale pasgeborenen na ART, dragen mogelijk levenslange gezondheidsrisico's met zich mee. Hoewel de veiligheid van ART van groot belang is, is onderzoek op dit gebied tot nog toe zeer beperkt. Dit proefschrift richt zich op de rol van ART in de slechtere humane perinatale uitkomst. We wilden meer inzicht krijgen in de etiologie en manieren vinden om de veiligheid van ART in de toekomst te verbeteren. Het eerste doel van dit proefschrift was om te onderzoeken of *in vitro* kweek van humane embryo's tijdens de eerste paar dagen van pre-implantatie ontwikkeling een effect heeft op de perinatale uitkomst. Het tweede doel van dit proefschrift was om te onderzoeken of ART een effect heeft op de epigenetische regulatie van de humane placenta.

In **hoofdstuk 2** werd het effect geëvalueerd van *in vitro* kweek medium op de humane perinatale uitkomst door de uitkomsten van eenling zwangerschappen te vergelijken na alternerend gebruik van twee commercieel verkrijgbare opeenvolgende mediasystemen. Data van een cohort van 294 levend geboren eenlingen verwekt na terugplaatsing van een vers embryo tijdens de IVF-behandeling, evenals data van 67 eenlingen verwekt na terugplaatsing van een ingevroren embryo (FET) en van 88 kinderen van 44 tweelingzwangerschappen na terugplaatsing van een vers embryo, werden geanalyseerd. *In vitro* kweek in medium van Cook resulteerde na terugplaatsing van verse embryo's in eenlingen met een lager geboortegewicht (gecorrigeerd gemiddeld verschil, 112 gram, $P = 0.03$), in meer eenlingen met een laag geboortegewicht <2500g ($P = 0.006$) en een laag geboortegewicht voor de zwangerschapsduur ≥ 37 weken ($P = 0.015$), in vergelijking met eenlingen geboren na kweek in medium van Vitrolife AB. Zwangerschapsduur ten tijde van de geboorte was niet gerelateerd aan het gebruikte medium (gecorrigeerd verschil, 0.05 weken, $P = 0.83$). Onder tweelingen in de Cook groep, werd een hoger verschil gevonden in het gemiddelde geboortegewicht tussen de tweelingen en meer geboortegewicht discordantie (>25% verschil). Onze bevindingen ondersteunden onze hypothese

dat kweekmedium de perinatale uitkomst van IVF eenlingen en tweelingen beïnvloedt. Een soortgelijke trend werd gezien bij eenlingen geboren na FET. Deze resultaten geven aan dat *in vitro* kweek een belangrijke verklarende factor zou kunnen zijn voor de slechtere perinatale uitkomst na ART.

In **HOOFDSTUK 3** werd de invloed van embryo kweekmedium op foetale groeipatronen onderzocht in het eerder beschreven cohort eenlingen verwekt na terugplaatsing van verse embryo's, met speciale aandacht voor het begin van de groei divergentie, omdat abnormale foetale groeipatronen een belangrijke risicofactor zijn voor de ontwikkeling van chronische ziekten op latere leeftijd. We analyseerden echo onderzoeken tijdens een zwangerschapsduur van 8 (n = 290), 12 (n = 83) en 20 weken (n = 206) en gebruikten eerste trimester serum markers [pregnancy-associated plasma protein-A (PAPP-A) and vrij β-hCG]. Bij een zwangerschapsduur van 8 weken, was er geen verschil tussen de op crown-rump lengte-gebaseerde en op eicelpunctie-gebaseerde zwangerschapsduur (ΔGA) (n_{VL} = 163, n_C = 122, gecorrigeerd gemiddeld verschil, -0.04 dagen, P = 0.84). Bij een zwangerschapsduur van 12 weken was er tussen de studiegroepen geen verschil in ΔGA, nekplooidikte (multiples of median, MoM) en PAPP-A (MoM). Echter het vrije β-hCG (MoM) ± SEM was significant verschillend (1.55 ± 0.19 in Vitrolife versus 1.06 ± 0.10 in Cook; P = 0.031). Bij een zwangerschapsduur van 20 weken werd bij het echo onderzoek in de Vitrolife groep (n = 115) een meer gevorderde zwangerschapsduur gezien overeenkomend met een toegenomen foetale groei, in vergelijking met de Cook groep (n = 91). Na correctie voor confounders was zowel het verschil tussen de zwangerschapsduur gebaseerd op 3 biparietale diameter daterings formules minus de werkelijke (op eicelpunctie-gebaseerde) zwangerschapsduur (gecorrigeerd gemiddeld verschil, +1.14 dagen (P = 0.04), +1.14 dagen (P = 0.04) en +1.36 dagen (P = 0.048), alsook de hoofdomtrek (HC) en trans-cerebellaire diameter (TCD) significant hoger in de Vitrolife groep (HC_{vl} 177.3mm, HC_c 175.9mm, gecorrigeerd gemiddeld verschil 1.8, P = 0.03; TCD_{vl} 20.5mm, TCD_c 20.2mm, gecorrigeerd gemiddeld verschil 0.4, P = 0.008). Concluderend waren de verschillen in foetale ontwikkeling na het kweken van embryo's in twee IVF media reeds aanwezig in het tweede trimester van de zwangerschap. Deze studie geeft aan dat de embryonale omgeving al belangrijk is voor de foetale ontwikkeling.

Hoofdstuk 4 beschrijft de kennis op het gebied van epigenetica in relatie tot de placentaire ontwikkeling en functie. Studies bij zowel dieren als mensen hebben steeds meer inzicht verschaft in het feit dat adequate epigenetische regulatie van zowel geïmprinte als niet-geïmprinte genen belangrijk is voor de ontwikkeling van de placenta. Verstoring van de epigenetische regulatie kan veroorzaakt worden door verschillende omgevingsfactoren, wat kan leiden tot een abnormale placentaire ontwikkeling en functie met mogelijke gevolgen voor maternale morbiditeit, foetale ontwikkeling en ziektegevoeligheid op latere leeftijd.

In **hoofdstuk 5** werd de mogelijke invloed van ART op de epigenetische regulatie van humane placenta's onderzocht. Placenta weefsel van 35 spontane zwangerschappen en 35 IVF/ICSI (5 IVF, 30 ICSI) zwangerschappen werd verzameld. De DNA methylatie patronen van een aantal opeenvolgende CpG's in de kernregio's van verschillende differentieel gemethyleeerde regio's (DMRs) die betrokken zijn bij de ouderspecifieke expressie van geïmprinte genen werden kwantitatief geanalyseerd, omdat deze betrokken zijn bij de placentaire en foetale groei en ontwikkeling. Zowel de promoter regio van *MEST* isoform α en β en de 6^{e} CTCF bindingsplaats binnen de *H19* DMR waren significant gehypomethyleerd in de IVF/ICSI groep. Dit verschijnsel werd consistent waargenomen op alle geanalyseerde CpG plaatsen en was niet beperkt tot enkele CpG plaatsen. De andere primaire en secundaire DMRs werden niet beïnvloed. De expressie van *H19* was verhoogd in de IVF/ICSI groep, terwijl de expressie van *IGF2* and *MEST* vergelijkbaar was. De eventuele klinische effecten van de waargenomen placentaire hypomethylatie op de ontwikkelingsprogrammering van de IVF/ICSI nakomelingen zijn nog onbekend. Of de hypomethylatie een aanpassing is van de placenta om de foetale toevoer te handhaven en het effect van omgevingsfactoren te verbeteren, of dat het een deregulering is die leidt tot een gestoorde ontwikkelingsprogrammering met of zonder verhoogde kwetsbaarheid voor ziekte, in overeenstemming met de developmental origins of health and disease (DOHaD) hypothese, dient verder onderzocht te worden.

Om het effect van ART op de epigenetische regulatie van de humane placenta verder te onderzoeken, werd in **hoofdstuk 6** een micro-array analyse (n = 10 in elke groep) uitgevoerd om genoombreed te onderzoeken welke paden gedereguleerd zijn in IVF/ICSI placenta's. De studie onthulde 13 significant

oververtegenwoordigde biologische paden betrokken bij de stofwisseling, immuunrespons, transmembraan signalering en controle van de celcyclus, en die waren grotendeels up-gereguleerd in de placenta monsters van de IVF/ICSI groep. Verder werd gezien het belang van geïmprinte genen voor de functie van de placenta, speciale aandacht gegeven aan verscheidene groeigerelateerde geïmprinte genen (*H19, IGF2, PHLDA2, CDKN1C, MEST* isoform α en β) door de expressie in een groter aantal monsters te onderzoeken middels kwantitatieve real-time PCR (qPCR) na standaardisatie ten opzichte van 3 huishoudgenen (*SDHA, YWHAZ* and *TBP*). Voor de genen die een verschil in expressie lieten zien, werd een kwantitatieve allel-specifieke expressie analyse uitgevoerd om verlies van imprinting (LOI) te onderzoeken, onafhankelijk van het mechanisme van imprinting. In deze prospectieve cohort studie konden respectievelijk 81 en 105 placenta's van IVF/ICSI en spontane zwangerschappen onderzocht worden na toepassing van enkele exclusie criteria zoals vroeggeboorte, intra-uteriene vruchtdood, het ontbreken van placenta monsters, zwangerschapscomplicaties of aangeboren afwijkingen. Speeksel monsters van beide ouders werden gebruikt om de ouderlijke allelen te onderscheiden. Zowel *H19* alsook *PHLDA2* liet een significante verandering in genexpressie zien, respectievelijk een 1.3-voudige ($P = 0.033$) en 1.5-voudige ($P = 0.002$) toename in mRNA expressie in de IVF/ICSI groep versus de controle groep. Echter, we vonden geen aanwijzingen dat deze verhoogde expressie te wijten was aan een verhoogde frequentie van LOI in de IVF/ICSI placenta monsters. Of deze epigenetische veranderingen in placenta's van IVF/ICSI zwangerschappen verband houden met de slechtere perinatale uitkomst na IVF blijft onbekend. Het is echter mogelijk dat deze verschillen de foetale ontwikkeling en langdurige patronen van genexpressie beïnvloeden, evenals de maternale fysiologie van de zwangerschap.

Hoofdstuk 7 geeft een algemene bespreking van de resultaten uit dit proefschrift in het kader van de recente literatuur. Conclusies van dit proefschrift, klinische implicaties en aanbevelingen voor toekomstig onderzoek worden besproken.

Hoofdstuk 8 biedt een valorisatie paragraaf om de mogelijke impact van dit proefschrift te overwegen en om de maatschappelijke en economische waarde te valoriseren.

Chapter 10

Co-authors and their affiliations

List of publications

Dankwoord

Curriculum Vitae

Co-authors and their affiliations

F. Busato, CEA-Institut de Génomique, Centre National de Génotypage, Laboratory for Epigenetics and Environment, Evry, France

Dr. E. Coonen, Department of Obstetrics & Gynaecology, GROW – School for Oncology and Developmental Biology, Maastricht University Medical Centre, Maastricht, The Netherlands

Dr. A.B. Coumans, Department of Obstetrics & Gynaecology, Maastricht University Medical Centre, Maastricht, The Netherlands

A. Daunay, Fondation Jean Dausset – CEPH, Laboratory for Functional Genomics, Paris, France

Dr. J.G. Derhaag, Department of Obstetrics & Gynaecology, GROW – School for Oncology and Developmental Biology, Maastricht University Medical Centre, Maastricht, The Netherlands

Dr. J.C.M. Dumoulin, Department of Obstetrics & Gynaecology, GROW – School for Oncology and Developmental Biology, Maastricht University Medical Centre, Maastricht, The Netherlands

Dr. L.M.T. Eijssen, Department of Bioinformatics-BiGCaT, Maastricht University, Maastricht, The Netherlands

Prof. dr. J.L.H. Evers, Department of Obstetrics & Gynaecology, GROW – School for Oncology and Developmental Biology, Maastricht University Medical Centre, Maastricht, The Netherlands

Prof. dr. J.P.M. Geraedts, Department of Genetics and Cell Biology, Maastricht University Medical Centre, Maastricht, The Netherlands

Prof. dr. J.A. Land, Department of Obstetrics & Gynaecology, University Medical Centre Groningen, Groningen, The Netherlands

Dr. ir. P.P.C.A. Menheere, Department of Clinical Chemistry, Maastricht University Medical Centre, Maastricht, The Netherlands

Dr. A.P.A. van Montfoort, Department of Obstetrics & Gynaecology, GROW – School for Oncology and Developmental Biology, Maastricht University Medical Centre, Maastricht, The Netherlands

Dr. L.L. Peeters, Department of Obstetrics & Gynaecology, University Medical Centre Utrecht, Utrecht, The Netherlands

Dr. L. Ponger, MNHN CNRS UMR7196, Paris, France, INSERM U565, Paris, France

Dr. L.J.M. Smits, Department of Epidemiology, University of Maastricht, Maastricht, The Netherlands

Dr. J. Tost, CEA-Institut de Génomique, Centre National de Génotypage, Laboratory for Epigenetics, Evry, France

Publications

Nelissen ECM, Dumoulin JCM, Busato F, Ponger L, Eijssen LMT, Evers JLH, Tost J, van Montfoort APA.
Altered gene expression in human placentas after IVF/ICSI
Hum Reprod. 2014 Dec;29(12):2821-31

Kleijkers SHM, van Montfoort APA, Smits LJM, Viechtbauer W, Roseboom TJ, **Nelissen ECM**, Coonen E, Derhaag JG, Bastings L, Schreurs IEL, Evers JLH, Dumoulin JCM.
IVF culture medium affects post-natal weight in humans during the first 2 years of life.
Hum Reprod. 2014 Apr;29(4):661-9.

Nelissen ECM, Van Montfoort APA, Smits LJM, Menheere PPCA, Evers JLH, Coonen E, Derhaag JG, Peeters LL, Coumans AB, Dumoulin JCM.
Среды для культивирования эмбрионов в программах ЭКО влияют на внутриутробное развитие человеческого плода уже во втором триместре беременности
Hum Reprod. 2013; 28(8): 2067-74.

Nelissen ECM, Van Montfoort APA, Smits LJM, Menheere PPCA, Evers JLH, Coonen E, Derhaag JG, Peeters LL, Coumans AB, Dumoulin JCM.
IVF culture medium affects human intrauterine growth as early as the second trimester of pregnancy.
Hum Reprod. 2013 Aug;28(8):2067-74.

Nelissen ECM, Dumoulin JCM, Daunay A, Evers JLH, Tost J, van Montfoort APA.
Placentas from pregnancies conceived by IVF/ICSI have a reduced DNA methylation level at the H19 and MEST differentially methylated regions.
Hum Reprod. 2013 Apr;28(4):1117-26.

Nelissen ECM, van Montfoort APA, Coonen E, Derhaag JG, Geraedts JPM, Smits LJM, Land JA, Evers JLH, Dumoulin JCM.
Further evidence that culture media affect perinatal outcome: findings after transfer of fresh and cryopreserved embryos.
Hum Reprod. 2012 Jul; 27(7):1966-76.

Nelissen ECM, van Montfoort APA, Dumoulin JCM, Evers JLH.
Epigenetics and the placenta
Hum Reprod Update. 2011 May-Jun;17(3):397-417.Review.

Dumoulin JCM, Land JA, van Montfoort APA, **Nelissen ECM**, Coonen E, Derhaag JG, Schreurs IEL, Dunselman GAJ, Kester AD, Geraedts JPM, Evers JLH.
Effect of in vitro culture of human embryos on birthweight of newborns.
Hum Reprod. 2010 Mar; 25(3):605-12.

Nelissen ECM, Lutgens SPM, Loo IHM van, Koek GH, Derhaag JG, Dunselman GAJ.
To do or not to do: IVF and ICSI in chronic hepatitis B virus carriers
Hum Reprod. 2009 July; 24(11):2676–8.

Nelissen ECM, Zwaan C de, Marcus MAE, Nijhuis JG.
Maternal cardiac arrest in early pregnancy
Int J Obstet Anesth. 2009 Jan;18(1):60-3.

Oral presentations

Nelissen ECM, Dumoulin JCM, Busato F, Ponger L, Eijssen LM, Evers JLH, Tost J, van Montfoort APA.
Veranderde genexpressie in humaan placentaweefsel na IVF/ICSI
Gynaecongres Papendal, Arnhem, November 2014

Nelissen ECM
The influence of human ART
Invited speaker Embryologenforum Austria, Innsbruck, September 2013.

Nelissen ECM
Effect van IVF kweek medium
Regionale refereeravond, Orbis Medisch Centrum Sittard, Mei 2012.

Nelissen ECM, van Montfoort APA, Menheere PPCA, Evers JLH, Peeters LL, Dumoulin JCM.
Effect of IVF culture medium on human fetal growth
Pre-selection Basic Science Award for oral presentation
ESHRE Annual meeting, Stockholm, Sweden, 2011

Nelissen ECM, Dumoulin JCM, Bras M, Schreurs IEL, Coonen E, Derhaag JG, Evers JLH, van Montfoort APA.
Placental gene expression of imprinted genes differs between IVF and non-IVF pregnancies
GROW Science day, Maastricht, November 2010

Nelissen ECM, van Montfoort APA, Coonen E, Derhaag JG, Evers JLH, Dumoulin JCM
Does the culture medium used during IVF treatment affect twin birth weight discordance?
ESHRE Campus symposium, Lisbon, Portugal, March 2008.

Nelissen ECM
Reanimatie in de zwangerschap
Regionale refereeravond, MUMC+ Maastricht, December 2006.

Poster presentations

Nelissen ECM, Dumoulin JCM, Bras M, Schreurs IEL, Coonen E, Derhaag JG, Evers JLH, van Montfoort APA.
Placental gene expression of imprinted genes differs between IVF and non-IVF pregnancies
GROW Science day, Maastricht, November 2010

Nelissen ECM, van Montfoort APA van, Coonen E, Derhaag JG, Evers JLH, Dumoulin JCM
In vitro culture affects the birthweight of human singletons
ESHRE Annual Meeting, Rome, Italy, June 2010.

Dankwoord

Promoveren is samenwerken. Ik heb dit enerverende traject tot een succesvol einde kunnen brengen dankzij mijn inspirerende promotor en co-promotoren, een solide basis van familie en vrienden en vele anderen, die ik wil bedanken voor hun enthousiaste inzet.

Allereerst een woord van dank aan alle zwangere vrouwen en hun partners die hebben deelgenomen aan de studies beschreven in dit proefschrift. Zonder jullie deelname was het onderzoek niet mogelijk geweest.

Prof. dr. J.L.H. Evers, geachte promotor, beste Hans. Eindelijk is het moment daar dat ik u officieel kan bedanken voor alles wat u de afgelopen jaren voor me hebt betekend! Ik kon altijd bij u terecht voor goed wetenschappelijk advies. Artikelen werden zeer vlot gereviseerd. Uw kritische blik zorgde er voor dat de kwaliteit van de stukken hoog gehouden werd. Daarnaast was u oprecht geïnteresseerd in de voortgang van het proefschrift en merkbaar trots bij behaalde resultaten. U gaf me alle vrijheid en veel vertrouwen gedurende mijn promotietraject. Ik ben trots op het eindresultaat. Bedankt voor alles.

Dr. J.C.M. Dumoulin, beste John. Jij was vanaf het prille begin betrokken. Dankzij jouw goede ideeën, wetenschappelijke ervaring en enorme doorzettingsvermogen is dit promotie onderzoek van de grond gekomen. Wat heb ik geboft met jou als mijn co-promotor. Ondanks je drukke agenda, stond je deur stond altijd open. Je behulpzaamheid en nauwkeurigheid ten aanzien van dBase was ongeëvenaard. Ook ken ik niemand die zo vriendelijk doch constructief feedback kan geven, helaas wel zonder gebruik te maken van de mogelijkheid om wijzigingen bij te houden in Word. Je was het niet altijd eens met mijn ideeën, maar meestal vonden we toch samen een oplossing om het geheel naar een hoger niveau te tillen. Dank voor dit leerzame traject.

Dr. A.P.A. van Montfoort, beste Aafke. Dankzij jouw enorme betrokkenheid, jouw positieve instelling en je enthousiasme, kon ik niet anders dan doorzetten. Het was een grote uitdaging voor ons beiden om het placenta onderzoek op te zetten. Van een methode om de placenta biopten af te nemen, te versturen en te bewerken tot het ontwikkelen van primers, niets was te gek. Dank voor je

vertrouwen in het onderzoek en in mij. Je begreep altijd precies wat ik bedoelde. Onze fantastische samenwerking, soms tot in de late uurtjes, was altijd erg nuttig en ook nog eens gezellig. Dank voor jouw vele op- en aanmerkingen. Ik heb veel van je geleerd.

Verloskundigenpraktijk Sittard, verloskundigenpraktijken rondom Maastricht, AIOS en verpleegkundigen van het Maastricht Universitair Medisch Centrum, Atrium Medisch Centrum te Heerlen en het Orbis Medisch Centrum te Sittard, bedankt voor jullie bijdrage aan het afnemen van de placenta biopten. Het was niet makkelijk voor jullie om dit na de bevalling ook nog te doen naast jullie drukke werkzaamheden.

Dear Jörg Tost, thank you for your great commitment and the reliable international cooperation. Sending our precious samples by post to France, was very exciting in the beginning. Although we have never met in person, it was a great pleasure working with you.

Aan alle mede auteurs: bedankt voor jullie inzet en commentaar.
Marij Janssen en Marion Meijs, bedankt voor al jullie administratieve ondersteuning van het onderzoek. Marijke Bras, Inge Schreurs, Sander Kleijkers, Roel Thijssen, Erika Timmer en Bieke Vanherle, dank voor jullie assistentie bij het verwerken van de samples. Trudy Sagis, je bent een goede en gezellige secretaresse. Dat we een vechtsport gemeen hebben, is toch wel heel bijzonder.

Geachte leden van de beoordelingscommissie (Prof. dr. C.E.M. de Die-Smulders, Prof. dr. J.J.H.M. Erwich, Prof. dr. S. Repping, Prof. dr. M.E.A. Spaanderman en Prof. dr. L.J.I. Zimmermann): ik dank jullie hartelijk voor het lezen en beoordelen van het proefschrift.

Ferdinand van Nispen, dank voor de mooie lay-out van mijn proefschrift.

Collega's van de afdeling IVF en het IVF-lab (Germaine, Laurence, Carla, Cecile, Marie-José, Erna, Tonny, Sylvia, Daniëlle, Katinka, Edith, Josien, John, Aafke, Sander, Marijke, Marij, Inge, Edith, Roel, Marion, Myriam, Sabina en PGD-afgevaardigden Marion D. en Jos), wat een fantastische ervaring om in zo'n hecht team te werken. We hebben lief en leed gedeeld en vaak krom gelegen

van het lachen. Ook al borgen jullie de chocolade na werktijd vaak goed op in de kast, meestal wist ik het toch te vinden en dat betekende niet veel goeds. Het waren mooie jaren waar ik regelmatig aan terugdenk.
Cèline Lardenoije, onze samenwerking op de IVF was heel kort, maar erg gezellig. Kim van Kaam, we hebben langer samengewerkt en zelfs menig ESHRE congres samen bezocht. Met z'n drieën (Aisha, jij en ik) in een te hete kamer in Rome vlak na mijn honeymoon was even wennen, maar we hebben wel flink gelachen. Wat goed dat je je hart gevolgd hebt en de switch hebt gemaakt naar de klinische genetica.

Lieve Aisha, mijn IVF buddy. In die 4 jaar hebben we veel meegemaakt. Naast de ups en downs op werkgebied, privé alsook in het onderzoek, genoten we enorm van onze tijd op de IVF. We behaalden destijds 4 keer achter elkaar de beste resultaten van Nederland. We konden veel zelf regelen en deden dat ook graag. Onze samenwerking was vanaf het begin erg goed, waardoor we een sterk team vormden dat elkaar aanvulde. We konden niet tegen elkaar uitgespeeld worden, want we wisten precies wat de ander gezegd had. Bedankt voor deze mooie tijd! Ik ben blij dat je mijn paranimf wil zijn. Jouw boekje komt er ook. Dat weet ik zeker. Hou vol!

Beste Dr. G.A.J. Dunselman, wat heb ik veel van u geleerd tijdens de '5-uurs show' en ook daarbuiten. U bent een zeer betrokken en deskundige gynaecoloog voor patiënten, maar ook voor mij als persoon had u altijd oog. Bedankt voor alle steun en adviezen.

Beste Jacques Maas, ik was altijd erg onder de indruk van je enorme gedrevenheid om kennis over te dragen en je kritische blik. Daarnaast ben je erg humoristisch en gezellig. Dat je met opgeheven blote benen achter het IVF bureau lag, als bijdrage aan de video voor onze bruiloft, was erg hilarisch en zal ik nooit vergeten.

Beste Ron van Golde, we hebben maar kortdurend samengewerkt op de IVF. Ik had samen met mijn collega Aisha de eer om je in te werken. Je hebt een warme persoonlijkheid en bent een enorme aanwinst voor de IVF.

Beste Janneke den Hartog, op de IVF hebben we net niet meer samengewerkt, wel toen je reeds gynaecoloog was. Ik heb je leren kennen als gedreven, nauwgezet, hartelijk en oprecht geïnteresseerd in mensen.
Onze opvolgers op de IVF, Celine Eggen, Guusje de Krom, Desiree Pattinaja en Heleen Zandstra, heel veel succes met alles en de meesten natuurlijk ook met jullie proefschrift. Zet hem op!

Gynaecologen en arts-assistenten van het Maastricht Universitair Medisch Centrum, bedankt voor de leerzame tijd, voor jullie interesse in mijn onderzoek en voor de schrijfstage van 3 maanden hetgeen een flinke sprong vooruit betekende. Collega AIOS, wat was het vaak gezellig in de assistentenkamer. De ene blunder was nog hilarischer dan de andere waardoor we vaak veel te laat naar huis gingen.

Maatschap gynaecologie en verloskunde van het Orbis Medisch Centrum te Sittard, bedankt voor het fijne opleidingsklimaat en jullie interesse in mij als persoon. Wat heb ik al ontzettend veel geleerd van jullie en ik ben ervan overtuigd dat dit nog veel meer wordt de komende tijd!

Collega-assistenten en oud-assistenten van het Orbis Medisch Centrum, Natascha, Lieke, Marjolein, Jonas, Violet, Fleur, Susanne, Rogier, Anouk, Nele en Anneloor, wat is het fijn om met jullie te werken in de kliniek. Iedereen werkt hard en is erg behulpzaam. Ook is er geregeld tijd voor gezelligheid en wordt soms tot in de late uurtjes of 's ochtends veel te vroeg bijgepraat op de app.

Verloskundigen, verpleegkundigen, polidames, fertiliteitsarts, POK- en OK-assistenten van het Orbis Medisch Centrum, dank voor de fijne werksfeer.

Studie vriendinnen van geneeskunde ('de geraniums'): lieve Annelieke, Esther, Lieke, Linda, Marieke, Reggy, Suzanne en Quirine bedankt voor de altijd gezellige feestjes, borrels, etentjes, uitstapjes en weekendjes weg. Het overlijden van Quirine heeft ons allemaal erg aangegrepen. Hierdoor proberen we zoveel mogelijk te genieten van elke dag. We zien elkaar niet zo vaak als we zouden willen wegens onze drukke werkzaamheden en uitbreidende gezinnetjes, maar als we afspreken is het altijd heerlijk bijpraten met chocolade als onmisbaar ingrediënt.

Mijn andere vriendinnen, Anouk, Jacqueline en Pauline, dank voor de gezellige momenten.

Lieve Nicole, mijn beste vriendin, bedankt dat ik met alles bij je terecht kan. We kennen elkaar al zo'n 24 jaar (wordt tijd voor een jubileumfeestje) door en door. We hebben al flink wat reisjes gemaakt samen naar de meest vreemde bestemmingen en iedere keer weer is het enorm gezellig. Zo kan zelfs het missen van het vliegtuig of overnachten op een bank op het vliegveld onze pret niet drukken. Ik zal de duo-massage die je voor ons regelde in Krakau nooit vergeten, maar voortaan kies ik toch liever zelf wie mijn masseur wordt. Ik verheug me op nog meer avonturen met jou nu het proefschrift af is. Bedankt dat je mijn paranimf wil zijn.

Lieve Inga en Dalida, mijn sisters, wat een genot was het om samen met jullie op te groeien. Zoveel gezelligheid en ook enige competitie onderling heeft het beste uit ons allemaal gehaald. Jullie waren altijd geïnteresseerd in de vorderingen van het boekje en wilden zelfs praatjes voor internationale congressen met me oefenen, ook al kostte het jullie soms moeite om te begrijpen waar het over ging. Nu het klaar is, is er weer meer tijd voor leuke dingen samen.

Lieve mama en papa, wat ben ik ontzettend dankbaar voor jullie onvoorwaardelijke liefde en steun! Jullie hebben me geleerd om hard te werken en je doelen na te streven. Daarnaast hadden jullie altijd een luisterend oor en het volste vertrouwen in alles wat ik deed en doe. Bedankt voor alles.

Lieve Francesco, mijn held. We zijn al zo lang bij elkaar dat ik bijna niet eens meer weet hoe het leven was zonder jou. Wat hebben we al ontzettend veel moois gezien en meegemaakt samen. Ook al had je soms het idee dat de laptop op de eerste plaats kwam tijdens het promotietraject, je hebt me altijd enorm gesteund. Door jouw humor, behulpzaamheid en relativeringsvermogen hebben we alle hordes samen overleefd. Ik ben ontzettend trots en blij dat we nu ons eerste kindje verwachten. Het getrappel in mijn buik tijdens het afronden van het proefschrift was erg speciaal. We gaan volop genieten van de toekomst.

Curriculum Vitae

Ewka Nelissen werd geboren op 14 februari 1979 te Sittard. In 1998 behaalde zij haar VWO-diploma aan het College Sittard. Hetzelfde jaar begon ze aan de studie Gezondheidswetenschappen aan de Universiteit van Maastricht. Na 2 jaar, in 2000, stapte ze na inloten over naar de studie Geneeskunde aan de Universiteit van Maastricht. In haar derde jaar volgde zij keuzeonderwijs Obstetrie in het Mount Hope Maternity Hospital, St. Augustine, Trinidad & Tobago. Tijdens haar co-schappen volgde zij een wetenschapsstage met als onderwerp 'Risk profiling of Lichen Sclerosus by methylation' bij de vakgroepen Gynaecologische oncologie en Pathologie. Tevens volgde zij een keuze co-schap Obstetrie en Gynaecologie in het St. Francis' Hospital, Katete, Zambia.

In 2006 behaalde zij Cum Laude haar artsexamen. Hierna werkte zij enkele maanden als arts-assistent niet in opleiding bij de afdeling gynaecologie/obstetrie in het Maastricht Universitair Medisch Centrum. Van november 2006 tot en met april 2011 werkte zij als IVF-arts/onderzoeker in het Maastricht Universitair Medisch Centrum. Tijdens deze periode startte zij met het promotieonderzoek, waarvan het resultaat voor u ligt, binnen het Maastrichtse onderzoeksinstituut GROW - School for Oncology & Developmental Biology, afdeling Obstetrie en Gynaecologie. Van mei 2011 tot en met december 2011 werkte zij als arts-assistent niet in opleiding bij de afdeling gynaecologie/obstetrie in het Orbis Medisch Centrum te Sittard. Vanaf januari 2012 is zij in opleiding tot gynaecoloog. De opleiding werd gestart in het Orbis Medisch Centrum te Sittard (opleider Dr. G.L. Bremer) en vanaf januari 2013 voortgezet in het Maastricht Universitair Medisch Centrum (opleiders Prof. dr. R.F.P.M. Kruitwagen, Dr. G.A.J. Dunselman). In juli 2014 keerde zij terug voor de opleiding naar het Orbis Medisch Centrum te Sittard.

Ewka is getrouwd met Francesco Cremers op 28 mei 2010. De geboorte van hun eerste kind wordt enkele weken na de verdediging van dit proefschrift verwacht.